UNIVERSIDADE NOVA DE LISBOA

Biochemical study of Polynucleotide Phosphorylase from the foodborne pathogen *Campylobacter jejuni*

Jorge Miguel Fonseca Casinhas

Dissertation presented to obtain the Master Degree in Medical Microbiology

Oeiras, October 2015

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Oeiras, October 2015

I – List of Communications

J. Casinhas, Rute G. Matos, Nabila Haddad and Cecília M. Arraiano. "Study of exoribonucleases in the foodborne pathogen *Campylobacter jejuni".* To be presented in the Congresso Nacional MicroBiotec'15, 10-12 of December of 2015, Évora University, Évora, Portugal. Poster.

II – Acknowledgements

I want to dedicate some words in my native language to all that supported me in the realization of this Master:

Gostaria de agradecer à Comissão Científica e Organizadora da sexta edição do Mestrado em Microbiologia Médica pelas qualidades e condições de ensino que proporcionaram durante estes últimos dois anos.

Agradeço também a todos os professores que leccionaram os módulos do ano curricular do Mestrado por toda a dedicação e apoio, bem como aos meus colegas de Mestrado pelos momentos vividos, pelo companheirismo e amizade que surgiu entre nós.

O meu sincero agradecimento à professora Cecília Arraiano por me ter dado a oportunidade de realizar o estágio de Mestrado no seu laboratório e com a sua equipa, que não só me fez crescer a nível intelectual, como também a nível pessoal.

Não posso deixar de agradecer a todas as pessoas do laboratório CMA, pelas palavras amigas, pelos momentos de diversão e pelo óptimo ambiente de trabalho que proporcionam.

Quero também fazer um agradecimento à minha co-supervisora Rute Matos por partilhar comigo a sua experiência, por apoiar-me com o seu conhecimento e por estar sempre disponível a ajudar-me com qualquer esclarecimento. Sem ela, esta etapa seria muito mais difícil de terminar.

Um agradecimento especial à minha namorada Marta por estar sempre presente, pela sua paciência em alguns momentos e por todo o apoio e carinho dispensado.

Por fim, quero agradecer aos meus pais, que sem o seu apoio nunca teria chegado aqui.

III – Abstract

Bacteria must adapt and rapidly respond to different environmental conditions. Ribonucleases are the enzymes responsible for the maturation and degradation of RNA molecules, enabling a fast adaptation of RNA levels to different environments. This adaptation is crucial to bacterial pathogens invade and establish inside the host.

Polynucleotide phosphorylase (PNPase) is a homotrimeric 3'-5' exoribonuclease that has both degradative and synthetic capabilities. It has been implicated in virulence in many human pathogens, namely in *Salmonella*, *Dichelobacter nodosus*, *Dickeya dadantii*, *Yersinia* and *Campylobacter jejuni*. *C. jejuni* is an important human foodborne pathogen, and is considered as the leading cause of human bacterial gastroenteritis worldwide. However, the information regarding RNA metabolism in this pathogen is limited. It is known that PNPase is essential for low-temperature cell survival, affects the synthesis of proteins involved in virulence and has an important role in swimming, cell adhesion/invasion ability, and chick colonization.

A better understanding about *C. jejuni* PNPase biochemistry and how it is influenced by physical and chemical factors is an approach to understand how this ribonuclease is involved in virulence.

In this work we have characterized the biochemical activity of PNPase from *C. jejuni*. We have overexpressed and purified *C. jejuni* PNPase and PNPase_ΔS1 and PNPase_ΔS1ΔKH mutants, and tested their activity and binding ability using synthetic RNA substrates. We have demonstrated that PNPase activity is regulated according to the temperature. Moreover, both degradative and polymeric activities are highly regulated in the presence of certain metabolites. We have also shown that both the KH and S1 domains of PNPase play critical roles in substrate binding and trimerization, with consequences for the activity of the protein.

III - Resumo

As bactérias devem adaptar-se e responder rapidamente a diferentes condições ambientais. As ribonucleases são as enzimas responsáveis pela maturação e degradação de moléculas de RNA, permitindo uma rápida adaptação dos níveis de RNA a diferentes ambientes. Esta adaptação é crucial para as bactérias patogénicas invadirem e estabelecerem-se dentro do hospedeiro.

A PNPase (*Polynucleotide phosphorylase*) é uma exoribonuclease homotrimérica com actividade 3'-5' e que para além de degradar RNA, também é capaz de sintetizar caudas heteropoliméricas. Esta enzima tem sido implicada na virulência em muitos patogénicos humanos, nomeadamente em *Salmonella, Dichelobacter nodosus, Dickeya dadantii, Yersinia e Campylobacter jejuni. C. jejuni* é um importante patogénico humano de origem alimentar e é considerado como a principal causa de gastroenterite bacteriana em humanos em todo o mundo. No entanto, a informação sobre o metabolismo do RNA em *C. jejuni* é limitada. Sabe-se que a PNPase é essencial para a sobrevivência das células a baixa temperatura, afecta a síntese de proteínas envolvidas na virulência e tem um papel importante na motilidade, adesão das células/capacidade de invasão e colonização das aves.

Para compreender como a PNPase está envolvida na virulência é necessário fazer uma análise bioquímica desta ribonuclease e ver como esta é influenciada por factores físicos e químicos.

Neste trabalho caracterizámos a actividade bioquímica da PNPase de *C. jejuni*. Sobre-expressámos e purificámos a PNPase, os mutantes PNPase_ΔS1 e PNPase_ΔS1ΔKH de *C. jejuni* e testámos a sua actividade e capacidade de ligação utilizando substratos de RNA sintéticos. Demonstrámos que a actividade da PNPase é regulada em função da temperatura. Além disso, verificámos que as actividades degradativas e de polimerização são altamente reguladas na presença de certos metabolitos. Mostrámos também que os domínios KH e S1 da PNPase desempenham um papel importante na ligação do substrato e na formação de trímeros, com consequências para a actividade da proteína.

IV – Table of Contents

V – List of figures

VI – List of Tables

VII – Abbreviations

A/U – Adenine / Uracil
APS – Ammonium persulfate
ATP – Adenosine triphosphate
bp - Base pair
BSA - Bovine serum albumin
C-di-GMP - Cyclic di-GMP
C-terminal - Carboxi-terminal
ddH_2O - Double-distilled water
DNA - Deoxyribonucleic acid
DNase - Deoxyribonuclease
dsRNA - Double stranded RNA
DSS - Disuccinimidyl suberate
DTT - Dithiothreitol
EDTA - Ethylenediamine tetraacetic acid
IPTG - Isopropyl-beta-D-thiogalactopyranoside
LA - Luria Agar
LB - Luria Broth
Mg^{2+} - Magnesium ion
Mn^{2+} - Manganese ion
N_2 - Nitrogen
N-terminal - amino-terminal
O.N. - Over night
OD - Optical density
Orn – Oligoribonuclease
PAGE - Polyacrilamide gel electrophoresis
PAP I - Poly(A) Polymerase I
PCR - Polymerase chain reaction
Pi - Inorganic phosphate
PMSF - Phenylmethylsulfonyl fluoride
PNPase - Polynucleotide Phosphorylase
ppGpp - Guanosine tetraphosphate
RNA - Ribonucleic acid
RNase- Ribonuclease
Rpm - Rotations per minute
rRNA - Ribosomal RNA
SDS - Sodium dodecyl sulphate
ssDNA - Single-stranded DNA
ssRNA - Single-stranded RNA
TB - Terrific Broth
TBE - Tris-borate-EDTA
TEMED - Tetramethylethylenediamine
tRNA – Transfer RNA
TTSS - Type three secretion System
UV - Ultraviolet
V – Volts
Wt - Wild-type

1. Introduction

1.1. The importance of the regulation of the RNA levels

Many of the biological processes that occur in all live organisms can not be totally understood if we do not have a detailed knowledge about RNA (1).

The gene expression level is determined mainly by the efficiency of transcription of RNA, the stability of mRNA, and the frequency of its translation into proteins.

The wide variation in mRNA stability in both prokaryotes and eukaryotes is the first evidence that decay rate controls gene expression (2,3). mRNA transcripts have limited but adjustable lifetimes, and their instability allows the modulation of the expression of genetic information according with cellular requirements (4-7). This principle holds true for multicellular organisms and for bacteria (8).

In prokaryotic organisms, the continuous mRNAs synthesis and degradation permit not only the metabolic changes that are required as cells grow and divide, but also a rapid adaptation to new environments and conditions (9).

1.2. RNA decay – Ribonucleases

The activity of ribonucleases is important to determine the levels of functional mRNAs in all living cells (2,9-12). Ribonucleases (RNases) are the enzymes which modulate the processing, degradation and quality control of all types of RNAs, and are key factors in the control of biological processes (2,12,13).

During RNA degradation, RNases do not only act just as “molecular killers”, but they act in agreement with the requirements of growth in adaptation to the environmental conditions. RNases act as a global regulatory network, monitoring and adapting the RNA levels to the cell needs (12,14,15). RNases also play an extremely important role in contributing to the recycling of ribonucleotides, and also carry out surveillance, by destroying aberrant RNAs that would produce detrimental proteins (12).

Bacteria utilize a large arsenal of ribonucleases, many of which are present only in certain bacteria (8). The most extensively studied bacteria in this field is *Escherichia coli*, where about 20 RNases have been identified so far (16).

Ribonucleases are divided into endoribonucleases, which cleave the RNA internally, and exoribonucleases, which cleave the RNA from one of the extremities removing the terminal nucleotides (12). They can act in association mediating the mRNA turnover (2,12,14).

1.2.1. Endoribonucleases

Endoribonucleases are the enzymes that cleave RNA internally by digesting phosphodiester bonds of the RNA molecule. They play an important role in mRNA metabolism and, in *E. coli*, the major endoribonucleases involved in mRNA degradation are RNase E and RNase III (8).

1.2.1.1. RNase E

RNase E, encoded by the *rne* gene, is an important endonuclease, essential for cell growth (17,18). Homologues of RNase E have been identified in 450 bacteria, archaea and plants (19).

E. coli RNase E is a single-stranded, nonspecific endonuclease with a preference for cleaving A/U-rich sequences (20,21). *In vitro* experiments have shown that purified *E. coli* RNase E prefers to cleave RNAs that are monophosphorylated at the 5' end (22). The efficiency of RNase E cleavage depends on the structure of the substrates and the accessibility of putative cleavage sites. Potential cleavage sites can be occluded by alternative secondary structures or by RNA-binding proteins (23).

The cellular levels and activity of RNase E are subject to complex regulation. The enzyme concentration in the cell is regulated by a feedback loop in which RNase E modulates the decay of its own mRNA, maintaining the level of the enzyme within a narrow range (24-27).

The active RNase E enzyme is a tetramer composed of four Rne monomers. RNase E also forms the core of a membrane associated macromolecular complex called the RNA degradosome (23) (see section 1.3.1.).

1.2.1.2. RNase III

RNase III, encoded by the *rnc* gene, belongs to the RNase III family. Members of this family are widely distributed among prokaryotic and eukaryotic organisms, sharing structural and functional features (28).

In *E.coli*, RNase III is not essential; however, the lack of this enzyme causes a slow growth phenotype (10,29).

RNase III is specific for double-stranded RNA and its role in RNA turnover has been associated with the removal of protective stem-loop structures that act as degradation barriers (12).

There are two different ways that RNase III can affect gene expression: a) as a dsRNA-processing enzyme, cleaving both natural and synthetic dsRNA (30), and b) when binds RNA without cleaving (31) (when dsRNA is not bound to the catalytic site), affecting RNA structure and modulating gene expression (32,33).

This endoribonuclease, like RNase E, has the ability to regulate its own synthesis with a specific cleavage near the 5' end of its own mRNA, removing a stem loop and promoting degradation by exoribonucleases (34,35).

1.2.1.3. RNase Y

RNase Y is an endoribonuclease which was recently identified in Gram-positive bacteria (36,37).

Although RNase E is absent in *Bacillus subtilis*, RNase Y is considered its functional analog (37). RNase E and RNase Y do not share sequence homology but are strikingly similar in function (38,39). Both enzymes prefer 5' RNA substrates with downstream secondary structures (22,37), have a major impact on the global RNA stability (37,40), are attached to the membrane (41-44), and interact with other proteins involved in RNA processing in a protein complex called the RNA degradosome

(36,45,46). *B. subtilis* RNase Y also interacts with RNases J1 and RNase III to control the abundance of total mRNAs (47).

1.2.1.4. RNase J1/RNase J2

RNases J1 and J2 have an important role in the maturation and degradation of RNA in *B. subtilis* (48). These enzymes have a large amino acid similarity and were initially characterized as endoribonucleases with *E. coli* RNase E-like cleavage specificity (49). However, besides its endonucleolytic activity, it was also reported that they can also act as 5'-3' exoribonucleases (50). RNase J1 has a robust 5'-3' exoribonuclease activity (51), but the equivalent activity of RNase J2 is just about a half (52).

Even though global mRNA stability is only weakly affected by RNases J1 and J2 (49), these enzymes play an extensive role in the maturation and degradation of many mRNAs (47,53,54), as well as in the maturation of rRNA (55) and small cytoplasmic RNA (56).

1.2.2. Exoribonucleases

Exoribonucleases are the enzymes that degrade RNA by removing terminal nucleotides in the 3'-5' direction or in the 5'-3' direction (16,57). These proteins act by releasing nucleotides that can be reutilized for the synthesis of new RNA molecules. In prokaryotes, most RNases cleave the RNA in the 3'-5' direction.

The prokaryotic exoribonucleases are divided in four families: RNB, PDX, DEDD and DHH (Table 1). The three first families are more abundant and important. For this reason we will only focus on those. This classification is based on structural characteristics or derived from sequence analysis (57).

Table 1: Prokaryotic exoribonucleases families (adapted from (58))

Family	Prokaryotic members	Catalytic mechanism	Comments
RNB	RNase II RNase R	Hydrolytic	These enzymes are processive in the 3' to 5' direction. Distribuited by all domains of life.
PDX	RNase PH PNPase Archeal exosomes	Phosphorolytic	One or two PH domains per protein. Associate to form ring structures containing 6 PDX domains.
DEDD	RNase D RNase T Oligoribonuclease	Hydrolytic	3' to 5' activity. Some members have sequence specificity (deadenylases). Family members can be processive or distributive. Distant family members include DNases and polymerases.
DHH	NrnA NrnB	-	Also called nano RNases. Decay polarity controversial. Distant family members include DNases and phosphatases.

In *E. coli*, the RNB family is exemplified by the 3'-5' processive exoribonucleases RNase II and RNase R. The members of RNB family can be ubiquitously found in the three domains of life and have a similar modular organization. They are characterized by the presence of a well-conserved RNB domain (59). This domain is exclusive to all RNase II-like enzymes and contains four highly conserved motifs (I–IV) with several invariant amino acids (60). They also include conserved N-terminal sequences which are variable in both length and sequence within the subfamilies, but most of them contain one or more RNA binding domains. Moreover, at the C-terminus, the presence of a S1 RNA-binding domain has been proposed for all RNase II family members (16,57).

Members of the PDX family of enzymes are phosphate dependent exoribonucleases that release nucleoside diphosphates as products of degradation. This family comprises the 3'-5' exoribonucleases PNPase and RNase PH from bacteria, and the core of the exosome in archaea and eukaryotes. These exoribonucleases share a hexameric architecture, although they may differ in organization and/or presence of additional subunits/domains (16,57).

The DEDD family includes both deoxyribonucleases (DNases) and RNases that are involved in various aspects of RNA metabolism and also DNA proofreading and repair. This family includes bacterial RNase D, RNase T, oligoribonuclease, and DNA

polymerases with proofreading activity as well as other DNA exonucleases (57,61). All proteins of this superfamily have a characteristic core comprised of four invariant acidic amino acids (DEDD) plus several other conserved residues distributed in three separate sequence motifs (62).

In *E. coli*, seven exoribonucleases participate in maturation, turnover, and quality control of RNA. These exoribonucleases are PNPase, RNase II, RNase R, oligoribonuclease (Orn), RNase D, RNase PH, and RNase T. However, only the first four exoribonucleases appear to accomplish all RNA degradative activity in the cell (63). RNase R, RNase II and PNPase are considering the three major 3'-5' processing exoribonucleases in *E. coli* (64).

1.2.2.1. RNase II

RNase II, encoded by the *rnb* gene, is a hydrolytic exoribonuclease that processively degrades single-stranded RNA substrates from the 3' end to the 5' end. Its activity is blocked by secondary structures (65,66).

In *E. coli*, RNase II is the major hydrolytic exoribonuclease, responsible for approximately 90 % of the total exoribonucleolytic activity in cell extracts (67).

Although RNase II degrading activity is sequence-independent, the most reactive substrate is poly(A) tails. The degradation of these poly(A) tails can act like a protection against the degradation of some RNAs by other exoribonucleases (PNPase and RNase R) that need a short tail to bind to the RNA molecules to be degraded (68-70).

RNase II expression is differentially regulated at the transcriptional and post transcriptional levels. It is transcribed by two promoters, P1 and P2 (71). The exoribonuclease PNPase was shown to modulate RNase II expression by degrading the *rnb* mRNA (72). Endoribonucleases RNase III and RNase E are also involved in the control of RNase II expression at the post-transcriptional level. RNase III does not affect *rnb* mRNA directly, but affects PNPase levels, and RNase E is directly involved in the *rnb* mRNA degradation (73). Furthermore, a *gmr* gene (gene modulating RNase II) was shown to affect the stability of RNase II and allow its regulation according to the growth conditions (74).

The 3D structure of *E. coli* RNase II and its RNA-bound complex have been determined. The structure corroborated all existing biochemical data for RNase II, and elucidated the general basis for RNA degradation by RNase II (59).

1.2.2.2. RNase R

RNase R, like RNase II, degrades RNA processively in the 3' to 5' direction. These enzymes also share structural properties, including 60% sequence homology (75). RNase R activity is also sequence-independent, but whereas RNase II is sensitive to secondary structures, RNase R, like no other known 3'-5' exoribonuclease, is capable of degrading highly structured RNA. However, it requires a single-stranded 3' overhang of at least 7 nucleotides in order to degrade dsRNA (75-78). RNase R was also shown to be a key enzyme involved in the degradation of polyadenylated RNA (75,79). Independently from its ribonuclease activity, RNase R possesses an intrinsic helicase activity (80). The helicase activity is important for effective nuclease activity against a dsRNA substrate, particularly at lower temperatures and with more stable duplexes (81).

RNase R is encoded by the *rnr* gene and is regulated at the post-transcriptional level mainly by RNase E, although RNase G may also participate (82).

The activity of RNase R is modulated according to the growth conditions of the cell and is induced under several stress conditions, namely in cold shock, starvation or oxidative stress, and has also been shown to be required for virulence (76,83,84). RNase R is a highly unstable protein in exponentially growing cells, but is stabilized in stationary phase and other stress conditions (85).

Clearly, only the resolution of the RNase R structure will allow a full understanding of its remarkable modes of action.

1.2.2.3. RNase PH

Together with PNPase, RNase PH belongs to the PDX family of exoribonucleases. However, while PNPase has an important function in mRNA degradation, RNase PH is involved in tRNA metabolism, namely in the processing of

tRNA precursors, in ribosome metabolism, and in the modification of the 3′ end of small RNAs, including M1, 6S, and 4.5S RNA (86-90).

RNase PH can act as a phosphorolytic RNase by removing nucleotides following the CCA terminus of tRNA and also as a nucleotidyltransferase by adding nucleotides to the ends of RNA molecules (87,91,92).

The active structure of the protein is actually a homohexameric complex, consisting of three RNase PH dimers. RNase PH has homologues in many organisms, which are referred to as RNase PH-like proteins. The part of another larger protein with a domain that is very similar to RNase PH is called an RNase PH domain (93).

RNase PH is present in bacteria, and in the core of the exosome (see section 1.3.2. Exosome) in archaea and eukaryotes.

1.2.2.4. PNPase

Polynucleotide phosphorylase (PNPase) was discovered in 1955 by Severo Ochoa and Marianne Grunberg-Manago. Their work on PNPase, an enzyme that catalyzes the polymerization of polyribonucleotides, led to the Nobel Prize (94).

The PNPase, from the PDX family, is an enzyme involved in RNA turnover in bacteria and eukaryotic organelles (95).

This protein is characterized bellow (section 1.5).

1.2.2.5. Oligoribonuclease

PNPase, RNase II, and RNase R degrading activities generate 5’-terminal oligonucleotides ranging from two to five nucleotides in length as reaction products (75,77), whose accumulation can be deleterious to the cell. These remnants oligonucleotides are converted into mononucleotides by oligoribonuclease (Orn). This enzyme, which belongs to the DEDD family, is essential in *E. coli*, being the only ribonuclease that can efficiently degrade oligonucleotides. Orn has an important role in replenishing the cellular pool of RNA precursors (96), while also prevents the misincorporation of these oligonucleotides at the 5’ end of new transcripts (97).

Orn is not present in all bacterial species. Some species that lack this enzyme have been shown to contain a distinct ribonuclease with similar properties (98,99).

1.3. Complexes of RNases

RNA degradation machinery often takes the form of multiprotein complexes. These complexes, or their individual components, can target specific gene products or affect the relative composition of different transcripts through differential decay rates (100).

1.3.1. Degradosome

The degradosome is a large multiprotein complex that can drive the energy-dependent turnover of mRNA. It is believed to act as a general RNA decay machinery in which the components of the degradosome cooperate during the decay of many RNAs (12).

The RNA degradosome is a high molecular-weight complex of four major and three minor protein components. The major components, under normal growth conditions, include RNase E, PNPase, ATP dependent RNA helicase B (RhlB), and the glycolytic enzyme enolase (45). The RhlB helicase, in the presence of ATP, unwinds the stem-loops making them accessible to PNPase digestion (36,101-103). Only RNase E, PNPase, and RhlB are required to reconstitute the activity of the RNA degradosome *in vitro* (104).

The minor components of the degradosome, include GroEL, DnaK and polyphosphate kinase, whose functions are still unclear (45).

The *E. coli* RNA degradosome is built on the C-terminal region of RNase E that shows high sequence variation among closely related bacteria. Its carboxy-terminal half binds PNPase, RhlB and enolase (105,106).

The *E.coli* mutants in which degradosome assembly is disrupted, have a slow-growth phenotype and altered metabolic profiles (107), resulting from inability to degrade a number of degradosome substrates (105).

The degradosome complex can suffer changes in composition depending on the growth or stress conditions. For example, in different temperatures, two different RNA helicases are known to associate with RNase E. Those changes affect its RNA target spectrum (108,109).

RNA degradosome is likely to be found throughout the Proteobacteria, although the composition of the associated proteins appears to be variable. For example, *Pseudomonas syringae* has a multiprotein complex containing RNase E, RNase R, and a DEAD-box protein, and *Rhodobacter capsulatus* has a multiprotein complex containing RNase E, two DEAD-box proteins, and the Rho transcription termination factor (110,111). The phylogenetic plasticity of noncatalytic region of RNase E appears to be associated with a functional plasticity in the composition of the RNA degradosome (112,113).

1.3.2. Exosome

The exosome is a multiprotein complex that emerges as a central 3'-5' RNA processing and degradation machineries in eukaryotes and archaea (114,115).

The archaeal nine-subunit exosome consists of four orthologs of eukaryotic exosomal subunits: the RNase PH-domain-containing subunits Rrp41 and Rrp42 form a hexameric ring with three active sites, whereas the S1-domain-containing subunits Rrp4 and Csl4 form an RNA-binding trimeric cap on the top of the ring (116).

Some archaeal exosomes can have a dual function: they can add tails to the RNA molecules or degrade them since they have both phosphorolytic and polyadenylating activity (117).

The structure of the archaeal nine-subunit exosome is very similar to the one present in Eukarya and to bacterial PNPase (118). Because archaeal exosomes also contain homologs subunits from eukaryotes, the core architecture of exosomes is probably conserved in evolution (119).

1.4. Schematic representation of mRNA degradation pathways in prokaryotes (conventional model)

In bacteria, mRNAs can be degraded by a number of mechanisms that do not act independently but in parallel, and that target different sites with different efficiencies. In prokaryotes there are several possible pathways by which the mRNAs molecules are degraded.

Studies using *E. coli* and *B. subtilis* have given a detailed knowledge about the mechanisms of RNA decay and maturation for Gram-negative and Gram-positive bacteria (120,121). Figure 1 summarizes these decay pathways:

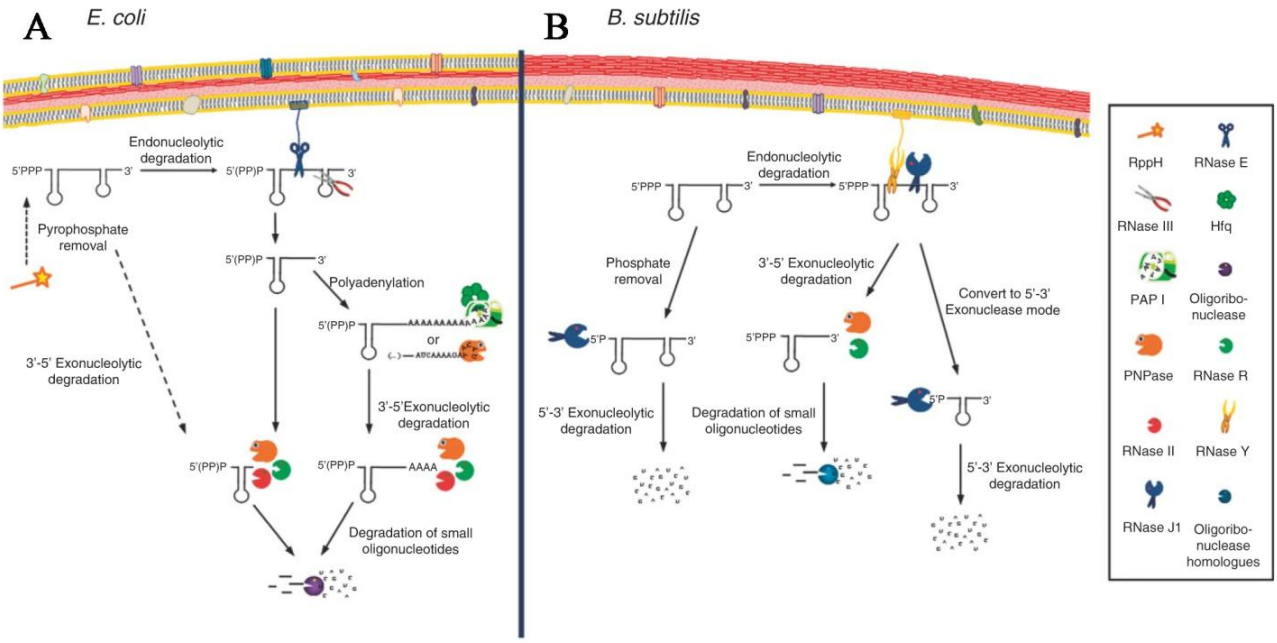


Figure 1: Schematic representation of mRNA degradation in *E. coli* and *B. subtilis* (122).

In *E. coli*, the decay of the majority of transcripts usually starts with an endoribonucleolytic cleavage by RNase E. A possible pathway for RNase E cleavage involves a primary cleavage by the RNA pyrophosphohydrolase RppH, which converts the 5'-triphosphorylated terminus of primary transcripts to monophosphate. RNase III can also initiate the decay of structured RNAs. After endoribonucleolytic cleavage, the breakdown products are ready for exoribonucleolytic digestion by any of the three main

exoribonucleases in this bacterium: RNase R, RNase II and PNPase. Exoribonucleolytic activity is promoted by the 3'-polyadenylation of substrates by PAP I and PNPase. The activity of PAP I, the main polyadenylating enzyme in *E. coli*, is modulated by the RNA-chaperone Hfq. A minor alternative pathway in the cell is the direct exoribonucleolytic degradation of full length transcripts. Exoribonucleolytic degradation releases short fragments which are subsequently degraded to mononucleotides by oligoribonuclease (Figure 1A).

In *B. subtilis*, transcripts can be degraded from the 5'-end through the exoribonuclease activity of RNase J1 after phosphate removal, or they can be first endonucleolytically cleaved by either RNase J1/RNase J2 or RNase Y. The breakdown products can be further degraded by the 3'–5' exonucleases, PNPase and RNase R (unprotected 3' ends), or by the 5'–3' exonuclease activity of RNase J1. The final products released by RNase R and PNPase are further degraded by the oligoribonuclease homologues in *B. subtilis* (Figure 1B).

1.5. A better understanding about PNPase

PNPase is encoded by *pnp* gene and is transcribed from two promoters (123). Its expression is negatively autoregulated at the post-transcriptional level. However, for the autoregulation occur, it is necessary the cleavage of the *pnp* message by RNase III (124-128). PNPase can also degrade mRNAs from other ribonucleases, namely the *rnb* mRNA (encoding RNase II) (72).

Although widely conserved in Bacteria and Eukarya, the *pnp* gene does not seem to be essential for survival at optimal temperatures, unless either *rnb* (gene coding for RNase II) or *rnr* (gene coding for RNase R) is also missing (129). However, PNPase is essential for bacterial growth in the cold (see section 1.5.2.) (126,130,131).

PNPase is an enzyme that has both degradative and synthetic capabilities. *In vitro*, it can degrade RNA from the 3' to 5' direction as well as add a heteropolymeric tail to the 3'end of the RNA molecule when inorganic phosphate (Pi) concentration is low (132-134). Instead of homopolymeric 3' poly(A) tails, known to destabilize mRNAs, heteropolymeric 3' tails are thought to render transcripts more stable (135). PNPase also catalyzes an exchange reaction between P-labeled inorganic phosphate and

the β-phosphate of nucleoside diphosphates. This reaction is apparently a result of combined polymerization and phosphorolytic reactions that occur under approximate equilibrium conditions (136-139).

In vivo, the degradative and polymeric activities of PNPase contribute to mRNA degradation. As an exoribonuclease, PNPase preferentially degrades RNAs with a minimal 3' single-stranded end of 7–10 nucleotides (140,141), although *E. coli* PNPase is known to be blocked by double-stranded RNA structures (66,140). As a polymerase, PNPase is capable of adding single-stranded adenine-rich tails that can facilitate the 3'-exoribonucleolytic degradation of structured regions of RNA (126,141). The tails added are predominantly adenosine, presumably because this is the most abundant nucleotide in all cells (142). It was observed that PNPase is responsible for residual RNA tailing in *E. coli* mutants devoid of the main polyadenylating enzyme, PAP I (Poly(A) Polymerase I) (70,143).

It was shown that *B. subtilis* PNPase can also degrade single stranded DNA in presence of manganese (Mn^{2+}) and low-level inorganic phosphate (Pi) (144). They suggest that PNPase reacts with broken DNA ends, either converting them from non-ligatable breaks to clean ends that can be sealed by DNA ligase or by adding non-templated single-stranded 3' tails that can then influence the choice of the repair pathway (144,145).

PNPase may exist as a single homotrimeric enzyme. However it can also associate with helicases or be part of a multiprotein degradative complex, the degradosome where it associates with RNase E, the RNA helicase RhlB, and enolase (see section 1.3.1.) (146). In such heteromultimeric associations, PNPase can degrade otherwise refractory double-stranded RNA regions in an ATP-dependent manner (45,101,102,146). In *E. coli*, PNPase has been localized both in the cytoplasm and associated with the ribosomes and cell membranes (in the degradosome complex (see section 1.3.1.)) (147,148).

1.5.1 Structure

The resolution of the crystal structures of PNPase from *E. coli (149), Streptomyces antibioticus (150),* and *Caulobacter crescentus* (151) showed that PNPase

has a homotrimeric organization with a ring-like architecture. The monomers exhibit a five-domain structure that is widely conserved (152,153). The domains are organized in two N-terminal RNase PH domains (RNase PH 1 and RNase PH 2, supposedly originated by a duplication event) connected by a α-helical linker, and two C-terminal domains, KH and S1 (Figure 2) (150,153,154).

The three monomers associate forming a homotrimer with a doughnut-shaped structure. The six RNase PH domains of the trimer form the core with a central channel where catalysis occurs. The three KH and S1 domains extend on the top immediately above the central channel and are required for proper RNA-binding (Figure 3) (147,150,154).

Figure 2: Linear representation of PNPase domains.

PNPase activity has been located in the second RNase PH-like domain, which exhibits higher sequence homology to RNase PH than the first core domain. However, RNase PH residues directly involved in the catalysis are not conserved in the second core domain of *E. coli* PNPase (147,155). Biochemical analysis of point mutants suggest that the catalytic activity is borne by the RNase PH 2 domain but also implicates the RNase PH 1 domain and the α-helical linker domain (125,126,147,149,156). The KH and S1 domains are responsible for substrate recognition and feed the RNA into the central channel. PNPase mutants lacking either the S1 or the KH domain retain phosphorolytic activity (125,149,156-158). However, the absence of these domains affect the stability off the trimeric structure (149), the products released and enzyme cycling, leading to a decreased turnover (156).

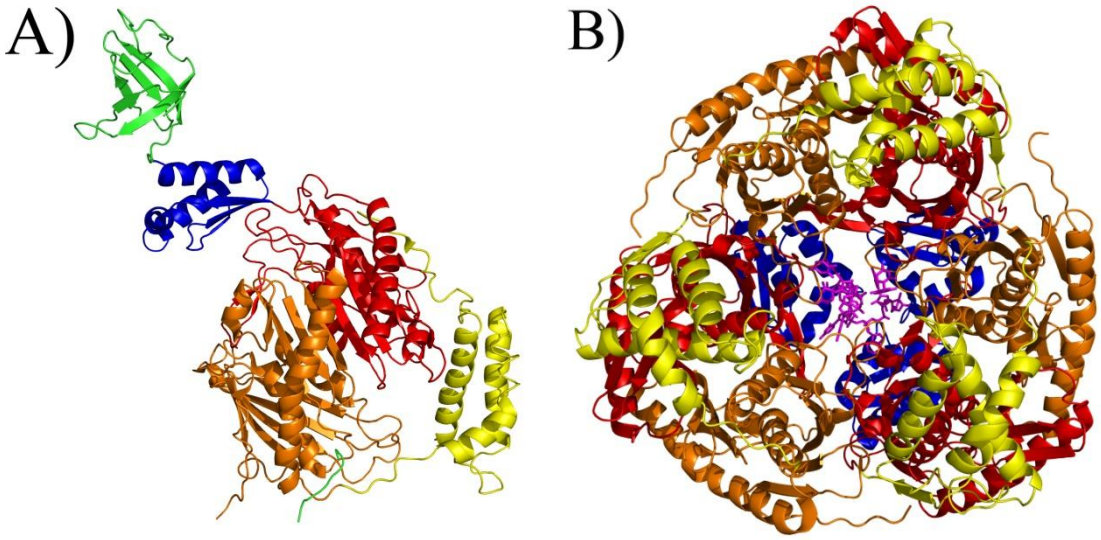


Figure 3: *C. crescentus* PNPase 3D crystal structure (images were obtained using PyMOL software (159)). Domains are coloured according to figure 2. **A)** View of PNPase crystal structure in the monomer organization (PDB 4AIM). **B)** View from the top of PNPase trimer (PDB 4AM3). RNA substrate (magenta) is observed in the central channel.

1.5.2. PNPase in cold shock conditions

With the decrease of the temperature, bacteria react with a specific response called the cold shock. In this response, a number of important changes occur in cellular physiology, which allows the cell to counteract these unfavorable changes, mostly by the selective production of a specific set of proteins (named cold induced proteins) (160). PNPase is one cold induced protein, and it has been reported that is essential for adaptation and growth at low temperatures in *E. coli, B. subtilis*, *Yersinia, Salmonella enterica, Streptococcus aureus,* and *C. jejuni* (156,157,161-166). In *E. coli*, it plays an essential role in stress adaptation by selectively degrading mRNAs for stress-response proteins (167).

In the cold adaptation phase, the *pnp* autogenous regulatory mechanism is transiently suppressed and the amount of *pnp* mRNA increases up to ten times (15,161,162).

It was shown, through KH and S1 mutant analysis, that the PNPase binding domains have an important role in cold adaptation. A complete deletion of both

domains is unable to complement the growth defect of a chromosomal Δ*pnp* mutant in the cold (126,158,164), suggesting that S1 and KH domains are essential to support growth in this conditions.

The bacteria cold adaptation capacity has medical significance, since pathogenic *Yersinia* and *C. jejuni* are contracted via the oral fecal route, and the fact that these pathogens can persist and grow at refrigerated/cold temperatures poses health threats in the form of yersiniosis and *C. jejuni* induced gastroenteritis (168).

1.5.3. Modulation of PNPase activity

The synergy between ribonucleases and cellular metabolic status illustrate a remarkable evolutionary convergence that implies an important contribution for organism fitness (169).

It was shown that cells lacking PNPase or degradosome-coupled PNPase activities differ in their metabolite concentrations when compared with parental strains (169). Moreover, some studies suggest that RNA degradative pathways communicate with central metabolism. The ability to modulate PNPase activity through metabolite binding provides a mechanism for wide-ranging regulation of RNA transcript levels in response to changes in the cellular environment (169).

1.5.3.1. ppGpp

The stringent response is a global bacterial response to nutritional or physical stress that is signaled by the alarmone ppGpp (guanosine 5'-diphosphate, 3'-diphosphate), and modulates transcription of up to 1/3 of all genes in the cell.

Accumulation of ppGpp results in a variation in gene expression pathways to allow bacterial survival under a multitude of unfavorable conditions. ppGpp function as a chemical messenger that allow bacteria to switch their metabolism from a "growth mode" to a "survival mode" (170). It results in a strong downregulation of genes for rRNAs and anabolic processes, and upregulation of genes involved in amino acid biosynthesis and stress survival (171-173). In *E. coli*, the alarmone ppGpp levels are known to be regulated by RelA and SpoT proteins (174).

The *C. jejuni* genome contains a gene, annotated as *spoT*, which exhibits strong homology to *relA* and *spoT* genes in other bacteria (175). It was shown that *C. jejuni spoT* gene is upregulated during infection of human epithelial tissue culture cells. It was also shown that the lack of SpoT is responsible for defective adherence, invasion, intracellular survival and growth under suboptimal CO_2 conditions (176).

Moreover, it was demonstrated that ppGpp inhibits both polymerization and phosphorolysis by *Streptomyces* and *Nomomurea* PNPase. Interestingly, this inhibition was not verified in *E. coli* PNPase (177,178).

1.5.3.2. ATP

Adenosine triphosphate (ATP) is a complex molecule that contains the nucleoside adenosine and a tail consisting of three phosphates. It is used in all cells as the primary energy currency (179).

Del Favero et al. (147) have studied how *E. coli* PNPase activity is modulated by ATP. They have concluded that ATP binds to PNPase and inhibits both its phosphorolytic and polymerization activities. The results present a clear-cut evidence (using both a model RNA and poly(A) as substrates) that *E. coli* PNPase binds and is inhibited by ATP. It was shown that the ATP-binding site is distinct from that of the substrates, as none of them could abolish or reduce the inhibitory effect even at saturating concentrations. These results strongly support the idea that the inhibition exerted by ATP on PNPase is a phenomenon of physiological relevance. Such a direct control by ATP highlights an unforeseen metabolic role of PNPase that connects RNA turnover and the energy charge of the cell (147).

1.5.3.3. Citrate

Decades of efforts to engineer metabolic pathways have revealed the complex behavior of metabolite concentrations and pathway fluxes in response to changing levels of enzymes.

Nurmohamed et al conducted a study where they show that the metabolite citrate affects the activity of *E. coli* PNPase. They also report that cellular metabolism is

affected by PNPase activity (169). They have demonstrated that citrate can bind directly to PNPase at physiological concentrations and when complexed with magnesium, inhibits the enzymatic activity of PNPase. The inhibition seen with magnesium-citrate is not only due to sequestering the required metal cofactor, but requires a specific ligand geometry (169). When citrate was tested predominantly in a metal-free form, the degradation and polymerization of the substrate were enhanced. It was suggested that citrate has two different binding sites: one where it is an inhibitor as the metal-bound form and one where it is an activator in its metal-free state (169).

1.5.3.4. Cyclic di-GMP

The second messenger cyclic diguanylic acid (c-di-GMP) is implicated in key lifestyle decisions of bacteria, including biofilm formation and changes in motility and virulence (180). More recently, this messenger has been implicated in RNA metabolism (181,182).

It was described that *E.coli* PNPase is a direct target of c-di-GMP. By *in vitro* assays with purified PNPase it was shown that the addition of c-di-GMP is responsible for the enhancement of two PNPase activities: polymerization and exchange reaction. As a conclusion, we can say that c-di-GMP can mediate signal-dependent RNA processing (183).

1.6. *Campylobacter jejuni*

C. jejuni is a Gram-negative microorganism, presently considered as the leading cause of human bacterial gastroenteritis worldwide (184-186).

Campylobacteriosis in humans is induced mainly by *C. jejuni* (about 90% of cases) (187-189) and it is characterized by serious common clinical symptoms: watery, bloody diarrhea, abdominal pain, fever, headache, and nausea. Moreover, in rare but significant cases, *C. jejuni* triggers autoimmune disease such as reactive arthritis or neurological disorders like Guillain-Barré syndrome (190-194).

The main reservoir of *C. jejuni* is the guts of avian species with up to 10^9 CFU (colony forming units)/g in faeces (193,195). Other farm animals and meat sources can

harbor the organism, and pets (especially young animals), including dogs, cats, hamsters, and birds, are potential sources of infection (196).

Transmission occurs by ingestion of contaminated food or water or by direct contact with fecal material from infected animals or people (196). Most of the infections (about 80%) are caused by improperly cooked poultry, untreated water, and unpasteurized milk transmission (197). Consumption and handling of poultry meat products are the major sources of campylobacteriosis in developed countries (193).

C. jejuni has specific microaerobic growth requirements, although, it is ubiquitous in aerobic environment. It has a great capacity to resist to different stresses during transmission, like dessiccation, temperature changes, osmotic stress and starvation. Also, it has the capacity to handle with the pH changes and the host innate immune response during human infection (198,199).

C. jejuni can adhere to intestinal cells, translocate through the intestinal barrier, and be highly invasive both *in vivo* and *in vitro* (200). Although it is not an obligate intracellular pathogen, the ability of a *C. jejuni* strain to invade host cells often correlates well with virulence *in vivo* (176,201). Intracellular survival may enhance its ability to evade the host immune system, cause relapse of the acute infection, and establish long-term persistent infections (202).

Previous studies have shown that *C. jejuni* grows optimally at a relatively high temperature between 37°C and 42°C (203,204), but their minimal growth temperature is in the range of 31°C to 36°C, and growth ceases abruptly around 30°C (204,205). At a lower temperature of 4°C, *C. jejuni* has the potential to survive on raw and cooked poultry, remaining at a viable but nonculturable stage for about 4 months (203,206). Biological activities, including protein synthesis, oxygen consumption, catalase activity, ATP generation, and motility, are still occurring at this temperature (205,206).

Considering that *C. jejuni* is a foodborne bacterial pathogen with impact on human health, it is important to have a more complete understanding about it. A better understanding about *C. jejuni* pathobiology, physiology, and regulation of the response mechanisms is necessary to facilitate appropriate intervention strategies in order to reduce *C. jejuni*-health-related problems (199,207,208).

1.7. Relation between exoribonucleases and virulence

Bacterial pathogens predominantly respond to environmental changes, such as entry into a host, by adapting their physiology through altered gene expression. The gene products that give a pathogen an enhanced chance of survival within the host are called virulence factors. Pathogens use a variety of different mechanisms to regulate virulence gene expression. Besides transcriptional control, several post transcriptional mechanisms that control mRNA stability are essential in controlling the expression of proteins that allow pathogenic bacteria to succeed within the host (209-212).

With an important role in the post transcriptional mechanisms, ribonucleases have been implicated as virulence-associated factors in many pathogens (168).

RNase R homologs have been identified in a wide range of species, and have been implicated in the virulence mechanisms of a growing number of pathogens.

It was shown, in *Aeromonas hydrophila*, that the absence of RNase R negatively influences the motility and attenuates virulence in mice (213). In *Mycoplasma genitalium*, RNase R is the only exoribonuclease present and is essential for cell survival (214). In *Shigella flexneri*, the causative agent of dysentery, RNase R homolog is extremely important for the expression of a variety of invasion factors, such as IpaB, IpaC, IpaD, andVirG (215). In *C. jejuni*, RNase R was shown to be active in a wide range of conditions. The plasticity exhibited by this enzyme may be crucial for the adaptation of *C. jejuni* during the infection process. In this same pathogen, RNase R was shown to be involved in adhesion and invasion of eukaryotic cells (208). RNase R is the only hydrolytic exoribonuclease present in *Legionella pneumophila*. This enzyme has a significant impact on viability at low temperatures and a role on competence induction of this bacterium (216).

These observations indicate that bacterial strains lacking RNase R exhibit an attenuated virulence phenotype when compared to their parental strains. This is probably connected to the fact that RNase R is a key player in RNA metabolism.

Besides the critical role in adaptation and growth at low temperature, PNPase also seems to be directly or indirectly involved in the expression of different virulence factors in some pathogenic bacteria.

In *Salmonella*, PNPase activity decreases the expression of genes from the pathogenicity islands SPI 1 (containing genes for invasion) and SPI 2 (containing genes for intracellular growth) (163,217). In *Dichelobacter nodosus*, PNPase acts as a virulence repressor in benign strains by decreasing twitching motility (218). In *Yersinia*, PNPase modulates the type three secretion system (TTSS) by affecting the steady-state levels of TTSS transcripts and controlling the secretion rate (164). This is probably the reason why the *pnp* deletion results in a less virulent strain in a mouse model (165). It has been shown that *C. jejuni* PNPase-less strain have swimming limitations, colonization delay and decrease of cell adhesion/invasion ability. Concluding, the *C. jejuni pnp* mutant is significantly less virulent than the wild-type strain (166,186). The multi-faceted roles played by PNPase make it an attractive protein to study in various model organisms (204).

1.8. Objectives

Ribonucleases are important factors in the regulation of bacterial virulence, enabling bacterial pathogens to invade and establish inside the host. Previous work has shown that *C. jejuni* PNPase facilitates survival of *C. jejuni* in low temperatures and favors swimming, cell adhesion/invasion, and chick colonization.

In this work we have purified *C. jejuni* PNPase (wild-type and truncated proteins lacking the RNA binding domains), and performed *in vitro* assays using a synthetic RNA substrate. With these experiments, we want to better understand the mechanism of action of this enzyme, how it is affected by physical and chemical factors and how it is involved in virulence.

The ultimate objective is to discover new strategies that will allow us to fight this pathogen and that may be extrapolated to other microorganisms.

2. Materials and Methods

All reagents and solutions are described on Appendix I (Table 11).

2.1. Bacterial strains

All bacterial strains used in this work are listed in Appendix I (Table 12) and were grown in LB medium at 37°C supplemented, when required, with appropriate antibiotic. Bacterial strains were stored in 10% glycerol at -80°C.

2.2. Preparation of competent cells (using rubidium chloride)

100 ml of LB medium were inoculated with *DH5α* or ENS134-3 cells from an overnight culture. Cells were grown with agitation at 37ºC and 150 rpm until the culture reached an OD_{600nm} between 0.4 and 0.6. The culture was incubated on ice for 15 minutes and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant was discarded and the pellet was gently resuspended in 40 ml of ice-cold TFB1. The suspension was incubated on ice for 15 minutes and centrifuged again at 5000 rpm for 10 minutes at 4°C. The pellet was resuspended in 4ml of ice-cold TFB2. The cells were incubated on ice for 15–60 minutes and then aliquot (100 µl each). Tubes were quick-frozen in liquid N_2 and stored at -80°C.

2.3. Transformation of competent cells (by heat shock)

Transforming DNA was added into *DH5α* or ENS134-3 competent cells (50 µl). It was incubated on ice for 30 minutes, heat shocked in a water bath at 42°C for 1 minute and returned to ice for 1 minute. LB medium (400µl) was added and cells were incubated at 37°C for 1 hour. After incubation, cells (100µl) were plated on LA medium plates (with appropriate antibiotic) and incubated overnight at 37°C.

2.4. Plasmid construction

The genes of interest (*pnp*_ΔS1 and *pnp*_ΔS1KH) from *C. jejuni* were obtained by PCR (Table 2 and 3).

The template used was plasmid pET-PNP_NB4, kindly provided by Doctor Nabila Haddad. This plasmid contains the *pnp* gene from *C. jejuni* with a N-terminal his-tag.

PCR reactions were performed with Phusion high-fidelity DNA polymerase (Finnzymes, Thermo Fisher Scientific®, Illkirch, France).

Genes *pnp*_ΔS1 and *pnp*_ΔS1KH were amplified using the primers Histag_Fw/DeltaS1_Rev and Histag_Fw/DeltaS1KH_Rev, respectively (Table 13 - Appendix II).

The primers DeltaS1_Rev and DeltaS1KH_Rev include on their sequences a stop codon at position 1719 and 1947 of the *pnp* gene respectively and a restriction site for enzyme *Bam*HI.

Table 2: Mix used for *pnp*_ΔS1 and *pnp*_ΔS1KH amplification.

Components	Amount
Master mix 2x (dNTPs; phusion; buffer)	12.5 µl
Forward Primer 10pmol/µl	10 pmol
Reverse Primer 10pmol/µl	10 pmol
DNA template (NB4)	10 ng
H_2O	Up to a final volume of 25 µl

Table 3: PCR program used for *pnp*_ΔS1 and *pnp*_ΔS1KH amplification.

Cycle Step	Temperature	Time	Number of cycles
Initial Denaturation	98°C	30 sec	1
Denaturation	98°C	10 sec	40
Annealing	55°C	30 sec	
Extension	72°C	45 sec	
Final Extension	72°C	5 min	1

The PCR reactions were visualized in an agarose gel, and the fragments with the correct size were purified from the gel with the kit NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL). These fragments, together with pET19b vector (Novagen) were double digested with the FastDigest restriction enzymes *Bam*HI and *Nde*I, as shown in table 5.

Table 4: Vector double digestion with *Bam*HI and *Nde*I.

Components	Amount
pET19b	500 ng
NdeI (FastDigest, Thermo Scientific)	1 µl
BamHI (FastDigest, Thermo Scientific)	1 µl
10x FastDigest Green Buffer	3 µl
H_2O	Up to 30 µl

Table 5: PCR fragment double digestion with *Bam*HI and *Nde*I.

Components	Amount
Insert (PCR product)	25 µl
NdeI (FastDigest, Thermo Scientific)	1 µl
BamHI (FastDigest, Thermo Scientific)	1 µl
10x FastDigest Green Buffer	3 µl
Final volume	30 µl

The restriction was performed at 37°C for 45minutes, and it was followed by an electrophoresis into an 0,7% agarose gel. The DNA fragments with the correct size were cut from the gel and purified using the kit NucleoSpin® Gel and PCR Clean-up (MACHEREY-NAGEL).

The purified DNA fragments were then ligated with the restricted plasmid. The ligation reaction was performed using T4 DNA ligase, at room temperature (approximately 22°C), for 10 minutes, according to table 6.

Table 6: Ligation of the double-digested PCR fragment with pET19b.

Components	Amount
Insert (Digested PCR product)	50 ng
Plasmid	10 ng
T4 DNA ligase	1 µl
10x T4 DNA ligase buffer	2 µl
H_2O	Up to 20 µl

After ligation, T4 DNA ligase was inactivated at 65°C for 10 minutes.

A fraction of 5µl from the ligation was used to transform competent *DH5α E. coli* cells following the protocol described above (see section 2.3.).

The transformants were selected with ampicillin (100 µg/ml). The colonies were grown overnight and plasmidic DNA was extracted using the kit NucleoSpin® Plasmid (MACHEREY-NAGEL). The positive clones were selected according to their restriction pattern using enzyme *AseI* (New Englands Biolabs®) (Figures 4 and 5).

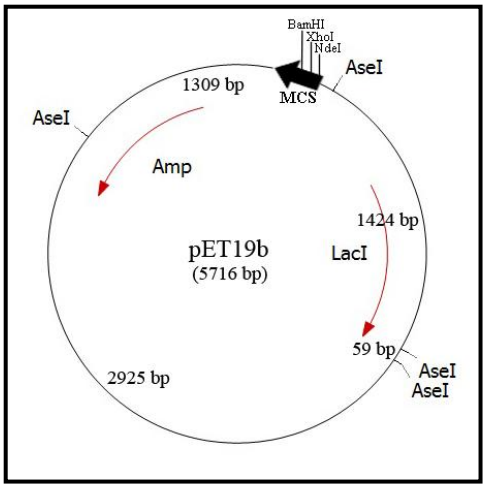


Figure 4: Schematic diagram of pET19b. Multiple cloning site (MCS) is represented by a black arrow, with the respective restriction sites (*BamHI*, *XhoI* and *NdeI*). *Amp* and *lacI* regions are represented by red arrows pointing the direction of transcription. *AseI* restriction sites and the size of the restriction fragments are indicated in the figure. The image was obtained by Clone Manager software program.

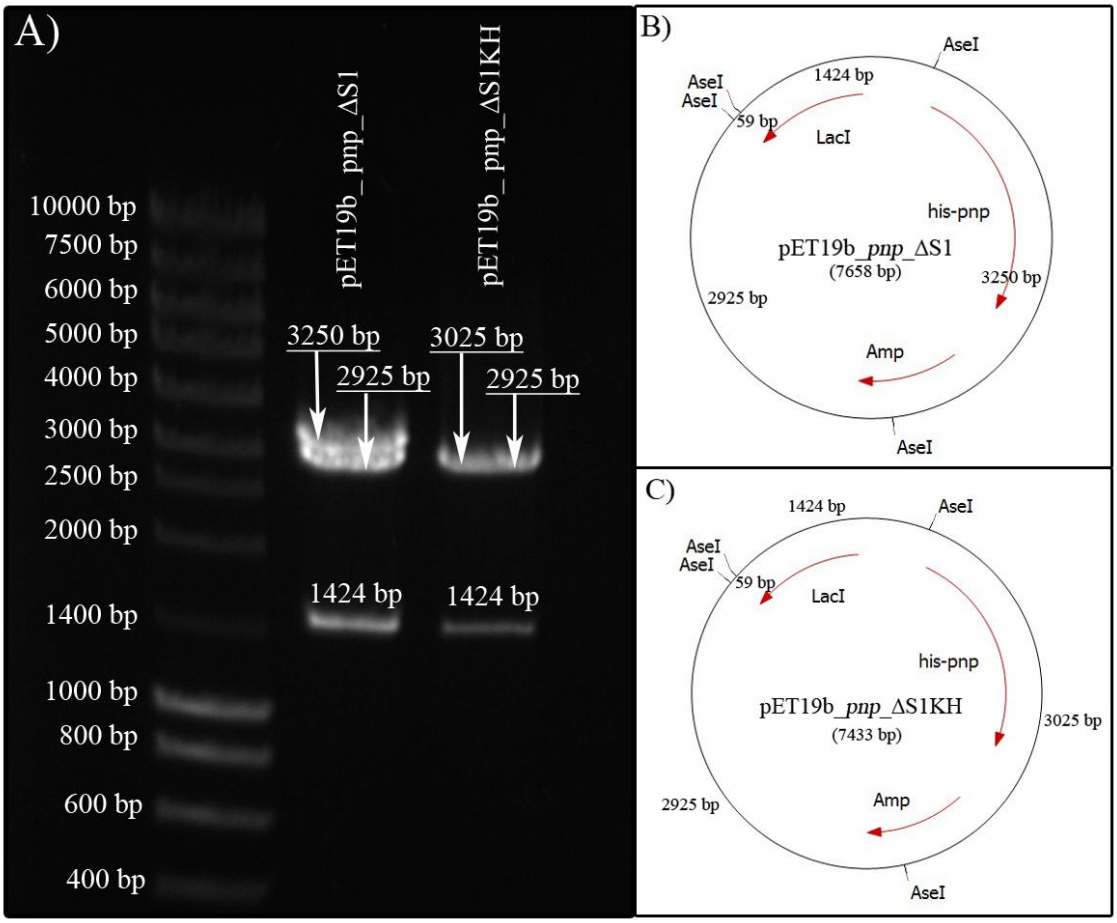


Figure 5: A) pET19b_ *pnp*_ΔS1 and pET19b_ *pnp*_ΔS1KH restriction with AseI enzyme, visualized on a 0.8% agarose gel. The DNA size marker is the commercial HyperLadder III. Bands were visualized by staining with ethidium-bromide. **B and C)** Schematic diagram of pET19b_ *pnp*_ΔS1 and pET19b_ *pnp*_ΔS1KH respectively. *His-pnp*, a*mp* and *lacI* regions are represented by arrows pointing the direction of transcription. AseI restriction sites and the size of the restriction fragments are indicated in the figure. The image was obtained by Clone Manager software program.

The positive constructions were confirmed by DNA sequencing at STAB Vida, Portugal. Transformants with the correct constructions were stocked at -80°C with 10% glycerol.

2.5. Protein overexpression and purification

To allow the expression of the recombinant proteins upon IPTG induction pET19b_ *pnp*_ΔS1, pET19b_ *pnp*_ΔS1KH and pET_PNP_NB4 constructions were transformed into ENS134-3 *E.coli* strain. Strain ENS134-3 derives from strain BL21(DE3). It contains the T7 RNA polymerase and has a transposable element inserted in *pnp* gene, causing its disruption (219).

Expression tests with wild-type PNPase were performed at different growth conditions. 1ml samples were collected after IPTG induction at the time points indicated in figure 6. The samples were centrifuged at 13000 rpm and the pellet was treated with BugBuster Protein Extraction Reagent (*Novagen*®) following the manufacturer's recommendations. All fractions were evaluated by SDS-PAGE analysis. The gel shown in figure 6 shows that the best conditions for PNPase expression is at 37°C overnight.

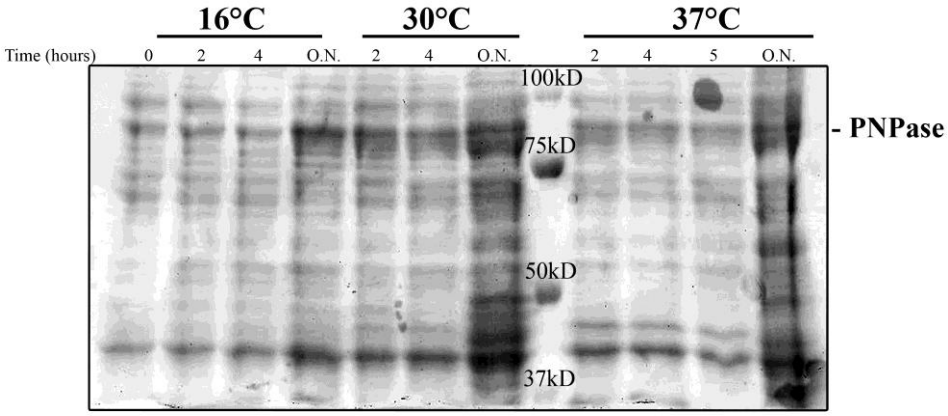


Figure 6: SDS-PAGE gel with samples of overexpressed *C. jejuni* wt PNPase, collected at different time points after IPTG induction in different growth temperatures. (O.N. – Over Night)

For the overexpression, transformed cells were grown at 37ºC and 180 rpm in 125ml of TB medium supplemented with ampicillin (100μg/ml) and kanamycin (50μg/ml) until they reach an OD_{600} of approximately 1. Protein expression was induced by addition of IPTG (0.5mM final concentration); induction proceeded for 20h at 37ºC. Cell cultures were centrifuged at 8000 rpm for 15 minutes at room temperature and the pellet was stored at -80°C.

2.6. Protein purification

2.6.1. HIS tag purification by affinity chromatography

The pellets obtained in section 2.5. were resuspended in 3 ml of Solution A (Table 11 - Appendix I) and 20 µl of PMSF (200mM) (protease inhibitor). The suspensions were transferred to 2 ml tubes filled with glass beads (1/4 of the volume). Cells were lysed using the FastPrep instrument at 6.5 m/s during 60 seconds. Samples were centrifuged at 14000 rpm for 30 minutes at 4°C and the supernatants were collected. The supernatants were incubated on ice with 0.2 µl of benzonaze (250 units/µl) (to degrade nucleic acids in the sample) for at least 1 h.

The histidine tagged recombinant proteins were purified by affinity chromatography, using ÄKTA FPLC™ System (*GE Healthcare*). The columns used were 1ml HisTrap Chelating HP (*GE Healthcare*) (Figures 7A, 8A and 9A).

The supernatants corresponding to each recombinant protein were loaded into the column previously equilibrated with Solution A, and elution was carried out by a linear gradient of Solution B (from 5mM to 500mM of imidazole). 10µl of each fraction was taken to an SDS-PAGE analysis (Figures 7B, 8B and 9B). The fractions containing our protein of interest were pooled together and concentrated using 50K MWCO Microcon (*Milipore*) columns.

***C. jejuni* PNPase wt**

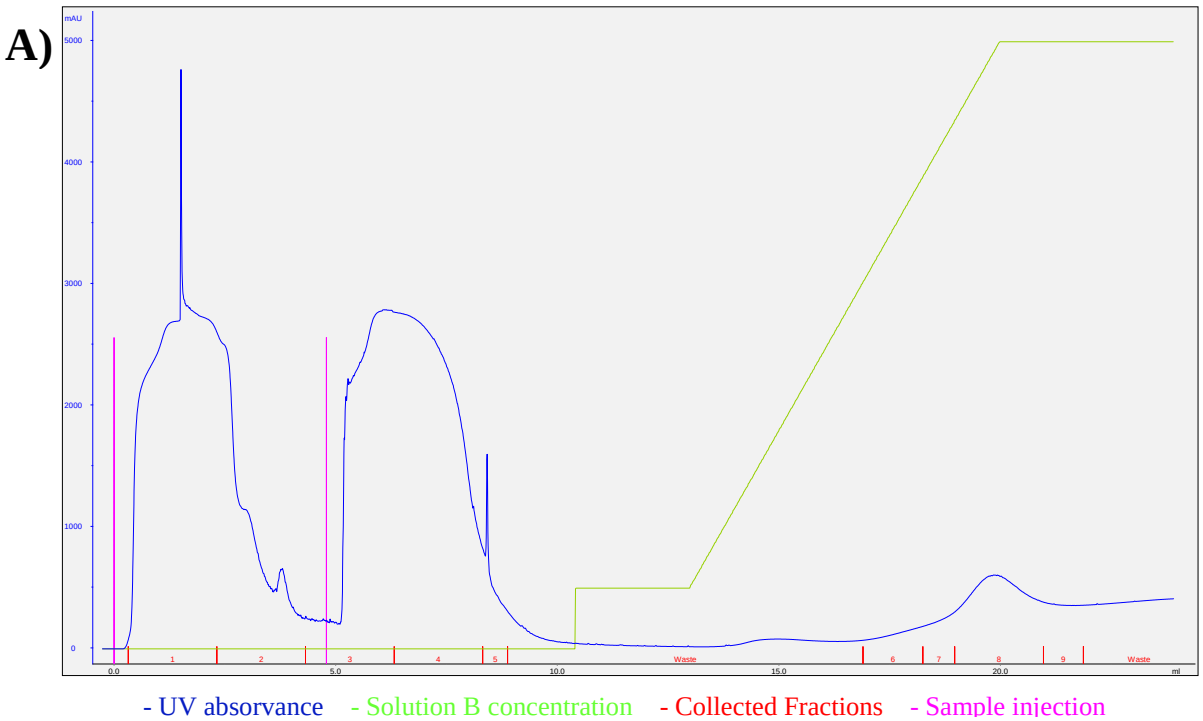
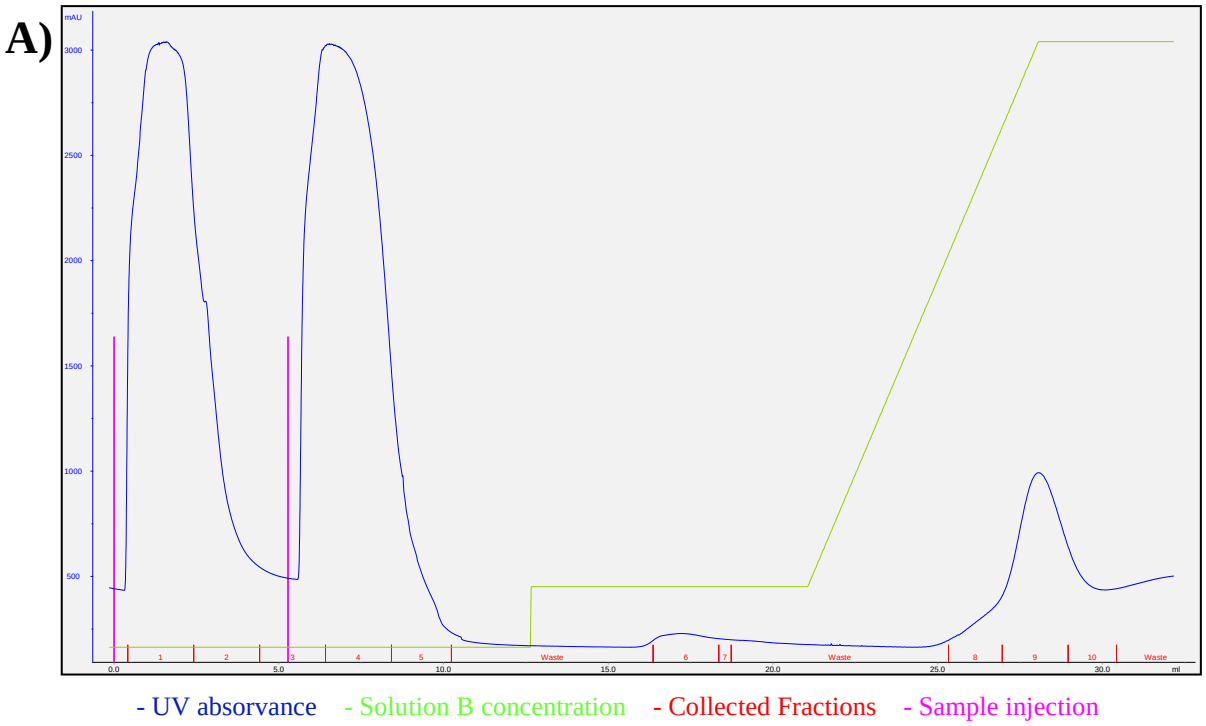


Figure 7: *C. jejuni* PNPase wild-type purification by affinity chromatography. **A)** Chromatogram obtained during protein purification. **B)** SDS-PAGE analysis of the fractions collected during the purification. Samples were denatured and separated in an SDS 8% polyacrylamide gel. Gel was stained with Comassie brilliant blue to visualize protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented.

***C. jejuni* PNPase _ΔS1**



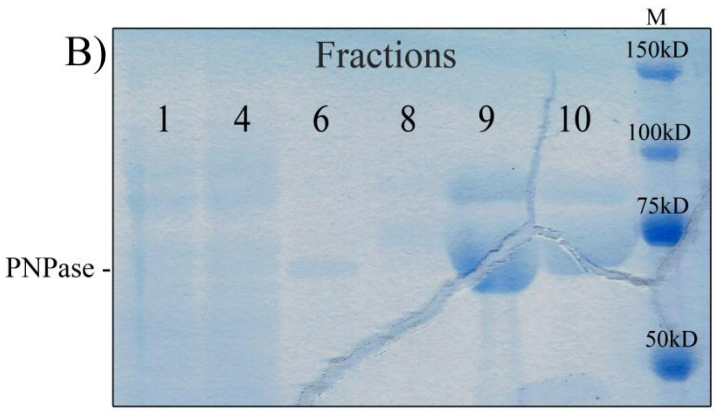
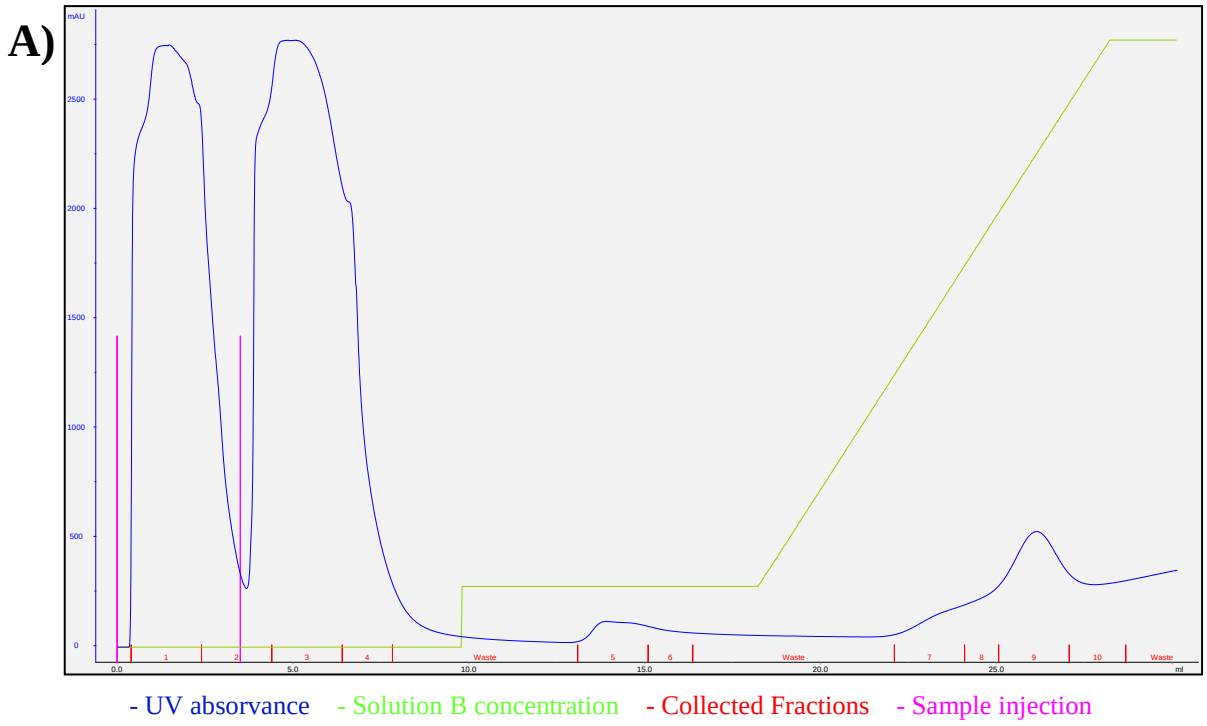


Figure 8: *C. jejuni* PNPase_ΔS1 purification by affinity chromatography. **A)** Chromatogram obtained during protein purification. **B)** SDS-PAGE analysis of the fractions collected during the purification. Samples were denatured and separated in an SDS 8% polyacrylamide gel. Gel was stained with Comassie brilliant blue to visualize protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented.

***C. jejuni* PNPase_ΔS1KH**



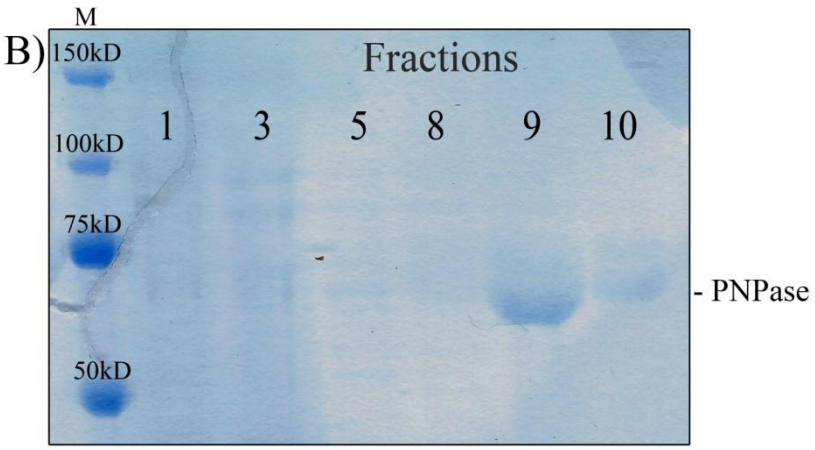


Figure 9: *C. jejuni* PNPase_ΔS1KH purification by affinity chromatography. **A)** Chromatogram obtained during protein purification. **B)** SDS-PAGE analysis of the fractions collected during the affinity purification. Samples were denatured and separated in an SDS 8% polyacrylamide gel. Gel was stained with Comassie brilliant blue to visualize protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented.

2.6.2. Gel filtration

In order to discard the contaminants, the purified proteins were subjected to a gel filtration step. Gel filtration is a chromatographic method that allows separating protein according to their size. For this, it was used a gel filtration column (Superdex 200) and the ÄKTA FPLC™ System (*GE Healthcare*). The column was equilibrated with two column volumes of water followed by two column volumes of Solution C. 100µl of the concentrated proteins were injected and passed through the column. The elution of the proteins was determined by monitoring the UV spectra at 280nm. Proteins were collected in fractions of 2ml. 10 µl of each fraction were analyzed by SDS-PAGE analysis. The fractions with the pure protein were stored at -20°C with 50% glycerol.

2.7. Determination of protein concentration using the Bradford method

The protein concentration in each fraction was determined using the Bradford method. 20 µl of each protein were taken and mixed with 1 ml of Bradford reagent (Bio-Rad®). The absorbance at 600nm was measured. The calculations for the determination of protein concentration were carried out according to a standard curve made with known concentrations of BSA.

2.8. Radioactive labeling of nucleic acids in the 5' end

The synthetic oligomers (Table 14 and 15 - Appendix II) were labelled at its 5'-end with γ^{32}P and T4 polynucleotide kinase (PNK). The reactions were incubated at 37ºC for one hour (Table 7).

Table 7: Components of the radioactive labeling of nucleic acids in the 5' end.

Components	Amount
Synthetic RNAs	1 µM
$\gamma^{32}P$	2 µl
10x Buffer PNK A	2 µl
PNK	0.5 µl
H_2O	Up to 25 µl

PNK was inactivated at 70°C for 10 minutes, and the oligomers were then purified using G25 column (*GE Healthcare*) to remove the nonincorporated nucleotides.

2.9. Activity Assays

Substrates were denatured at 80°C for 10 minutes and incubated at 37°C for 30 minutes to acquire its native conformation.

Exoribonucleolytic reactions were carried out in a final volume of 15µl (Table 8) containing 15 nM of substrate (Poly(A) (Table 14 – appendix II)), 1M Tris pH 8, 100mM DTT, 1M $MgCl_2$, 1M KCl and 0.5M NaH_2PO_4. The amount of each enzyme added to the reaction was adjusted for each experiment and is indicated in the respective figure.

Polymerization reactions were carried out in a final volume of 15µl (Table 8) containing 15 nM of substrate (16ss (Table 14 – appendix II)), 1M Tris pH 8, 100mM DTT, 1M $MgCl_2$, 1M KCl and 1mM ADP. The amount of each enzyme added to the reaction was adjusted for each experiment and is indicated in the respective figure.

Reactions were started by the addition of the enzyme and incubated at the selected temperature (4, 30, 37 or 42ºC). Samples of 3µl were withdrawn at the time points indicated in the figures, and the reaction was stopped by adding formamide-containing dye supplemented with 10 mM EDTA. Reaction products were denaturated for 5 minutes at 100ºC and resolved in a denaturing 20 % polyacrylamide/7M urea gel at 2000V for approximately three hours (until bromophenol blue migrate ~22 cm).

The gel was exposed on a phosphor screen for approximately 24 hours and the radioactive substrate was detected by phosphorimaging using the STORM™ imager (*Molecular Dynamics*).

The exoribonucleolytic activity of the enzymes was determined by measuring and quantifying the disappearance of the substrate in several distinct experiments in which the protein concentration was adjusted in order that, under those conditions, less than 25% of substrate was degraded. Quantifications were done using the *ImageQuant 5.0*® software (*Molecular Dynamics*). Each value obtained represents the mean of at least three independent assays. The specific activity of each enzyme is given as the nM of substrate consumed per minute per nM of protein.

Table 8: Components of activity assays reactions.

Components	Amount
Substrate	9 µl (15nM)
Activity buffer 5x	3 µl
Enzyme	3 µl
Final Volume	15 µl

2.10. Binding Assays

Binding assays reactions were performed using poly(A) substrate. This RNA was denatured at 80°C for 10 minutes and incubated at 37°C for 30 minutes to acquire its native conformation. The reactions were carried out in a final volume of 10 µl (Table 9) containing 10 nM of substrate, 1M Tris pH 8, 100mM DTT, 1M KCl and 0.5M EDTA. Mixtures containing increasing concentrations of each enzyme were incubated for 10 minutes at 37°C, 2 µl of loading buffer (6x) were added and the RNA-protein complexes were fixated by UV crosslinking at 120 x 100µJ/cm^2 for 3 minutes using UVC 500 CrossLinker (*Amersham Biosciences*) (220). The samples were analyzed in a 6% non-denaturing polyacrylamide gel, which was run in a cold room at 200V for 4 hours. The gel was exposed on a phosphor screen for approximately 24 hours, and the radioactive substrates were detected by phosphorimaging using the STORM™ imager (*Molecular Dynamics*).

Table 9: Components of binding assays reactions.

Components	Amount
Substrate	6 µl (25nM)
Binding buffer 5x	2 µl
Enzyme	2 µl
Final Volume	10 µl

2.11. Cross-linking of *C. jejuni* PNPase wt and mutants

In order to verify if PNPase wt and mutants are able to trimerize, the purified proteins were incubated with increasing concentrations of DSS (disuccinimidyl suberate). The cross link reactions were carried out in a final volume of 10µl, including 0.5 µg of purified protein, 10 mM Hepes pH 7.4, 250 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT (dithiothreitol) and increasing concentrations of DSS (1–10 µg) (Table 10).

The samples were incubated at room temperature for 20 minutes and quenched by adding 1 µl of Tris–HCl 1 M pH 7.5 and 10µl of NuPAGE® LDS Sample Buffer. Samples were boiled for 5 minutes, and then evaluated in a 8% SDS-PAGE gel.

Table 10: Components of cross-linking reactions.

Components	Amount
Purified protein	6.9 µl (0.5µg)
Cross linking buffer 5x	2 µl
DSS	1.1 µl
Final Volume	10 µl

3. Results

This section is divided in two parts. The first part includes the purification of *C. jejuni* wild-type and mutated PNPases, namely PNPase_ΔS1 and PNPase_ΔS1KH. In this part we also examine the capability of trimerization of all these proteins. The second part presents the characterization of all these proteins using *in vitro* assays in order to study the *C. jejuni* PNPase exoribonucleolytic activity, binding and polymerization activity.

3.1. Purification and trimerization capability of *C. jejuni* PNPase WT, PNPase_ΔS1 and PNPase_ΔS1KH.

The pET-PNP_NB4 (provided by Prof. Nabila Haddad), was used in this work to overproduce the wild-type PNPase. The pET-19b, used in the construction of pET_PNP_NB4, was also the expression vector to overproduce the PNPase_ΔS1 and PNPase_ΔS1KH proteins. This plasmid is a cloning option available within the pET vectors, which provides a convenient way for a high-level production of proteins. Briefly, it is a high copy plasmid that contains a T7 promoter and a lac operator both located upstream of the insert gene. Thus, the insert gene is not transcribed unless the T7 RNA polymerase is present. It carries an N-terminal His-Tag upstream of the inserted gene that will allow the purification of the expressed protein. It also has an enterokinase site that allows the removal of the His-tag. Finally, it has a T7 terminator located downstream of the inserted gene.

For protein expression, the *E. coli* ENS134-3 was used. This strain was kindly obtained from Doctor Marc Dreyfus, École Normale Supérieure, Paris, France. It is an PNPase-less strain derivative of *E. coli* BL21(DE3) that contains the T7 RNA polymerase gene under control of the IPTG-inducible lac promoter. This strain was used instead of a normal *E. coli* BL21(DE3) strain to ensure the purification of homotrimers.

After the first step of purification with a His Trap column (as described in chapter 2.6.1.) the concentrated proteins were purified by gel filtration. This technique allows not only to discard any contaminant, but also to distinguish between the monomeric, dimeric and trimetric forms (Figures 10,11 and 12). The column used in gel

filtration (Superdex 200) was selected according to the fractionation size range so that the expected molecular weight of our proteins falls approximately in the middle of the range for this column. The Superdex column has a high resolution in the separation of proteins, peptides or other biomolecules according to the size, with a range between 10 kDa and 600 kDa.

In each chromatogram obtained after gel filtration it is possible to observe different peaks that correspond to the different forms of the protein. The first peak corresponds to PNPase in the trimeric form, the second corresponds to PNPase in the dimeric form and the third corresponds to PNPase in the monomeric form.

By interpreting the amplitude of the peaks in the purification of each protein, it is possible to see that for *C. jejuni* PNPase wt (Figure 10A) the trimeric form is the most abundant one. In fact, the SDS-PAGE gel (Figure 10B) showed a higher amount of purified protein in the fractions that correspond to the trimeric form (fractions 2 and 3). In the *C. jejuni* PNPase_ΔS1 chromatogram (Figure 11A) it is visible that the monomeric form is the most abundant. However, it also has a significant amount of protein in the dimeric and trimeric form. The SDS-PAGE gel (Figure 11B) showed a higher amount of purified protein in the fractions that correspond to dimeric and monomeric forms (fractions 5 to 12). In the *C. jejuni* PNPase_ΔS1KH chromatogram (Figure 12A) it is visible that the monomeric form is the most abundant one. The dimeric and trimeric forms are present, but in a reduced manner when compared with the other proteins. In the SDS-PAGE gel (Figure 12B) it is possible to observe that the higher amount of protein is present in the fractions that correspond to the monomeric form (fractions 8 to 12).

***C. jejuni* PNPase wt**

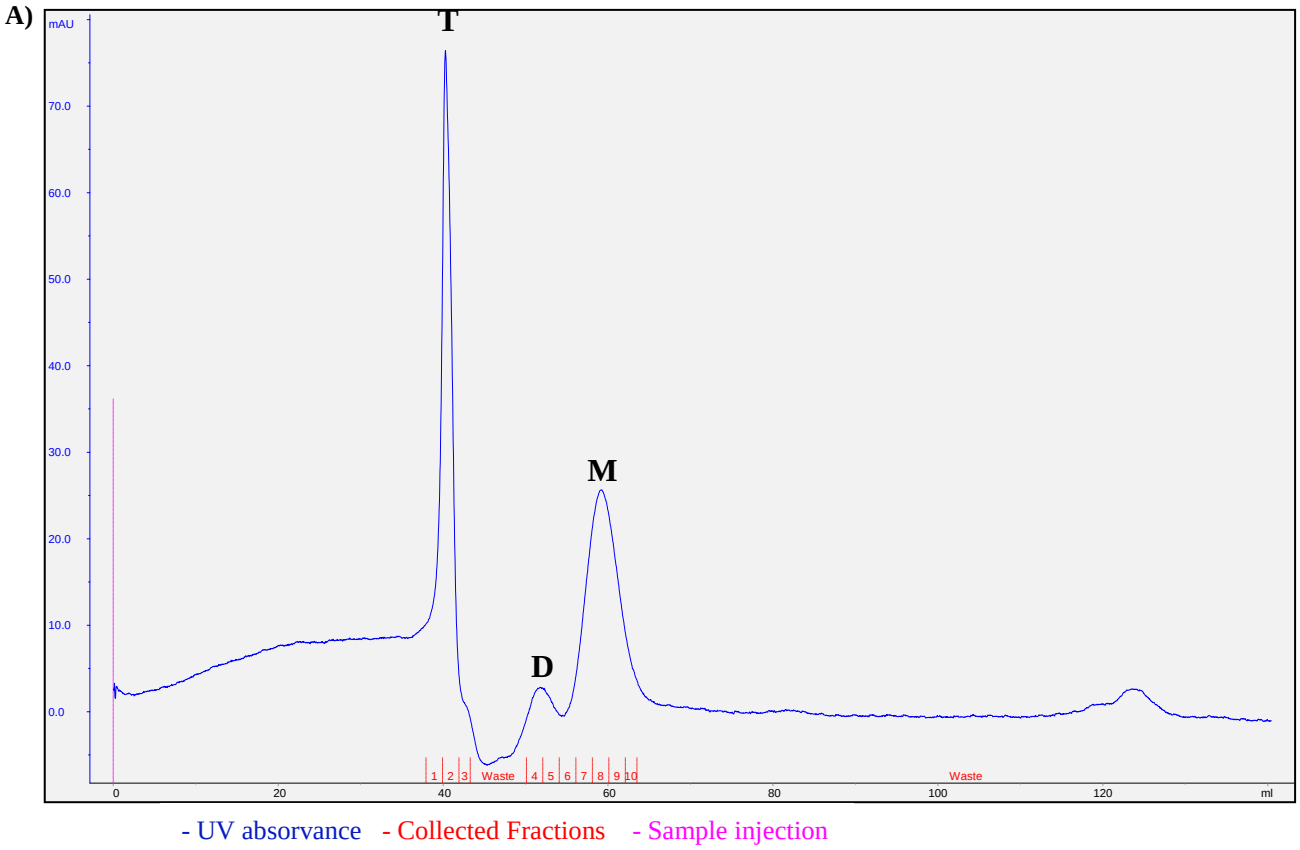


Figure 10: Gel filtration of *C. jejuni* PNPase wild-type. **A)** Chromatogram obtained in the protein purification. The first peak corresponds to PNPase in the trimeric form (T), the second corresponds to PNPase in the dimeric form (D) and the third corresponds to PNPase in the monomeric form (M). **B)** SDS-PAGE analysis of the fractions collected during gel filtration. Samples were denatured and separated in an SDS 8% polyacrylamide gel. Gel was stained with Comassie brilliant blue to visualize the protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented.

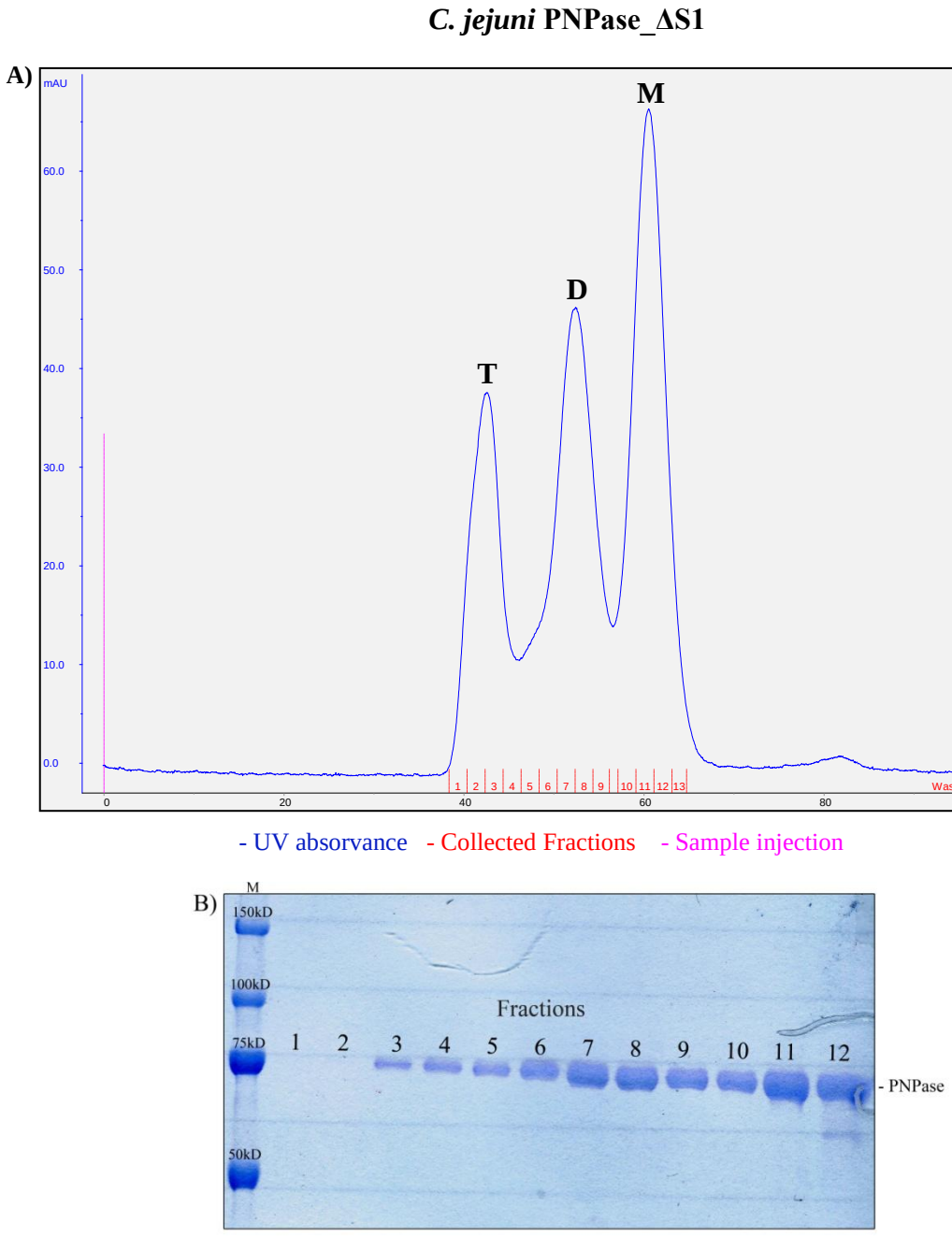


Figure 11: Gel filtration of *C. jejuni* PNPase_ΔS1. **A)** Chromatogram obtained in the protein purification. The first peak corresponds to PNPase_ΔS1 in the trimeric form (T), the second corresponds to PNPase_ΔS1 in the dimeric form (D) and the third corresponds to PNPase_ΔS1 in the monomeric form (M). **B)** SDS-PAGE analysis of the fractions collected during gel filtration. Samples were denatured and separated in an SDS 8% polyacrylamide gel. Gel was stained with Comassie brilliant blue to visualize the protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented.

***C. jejuni* PNPase_ΔS1KH**

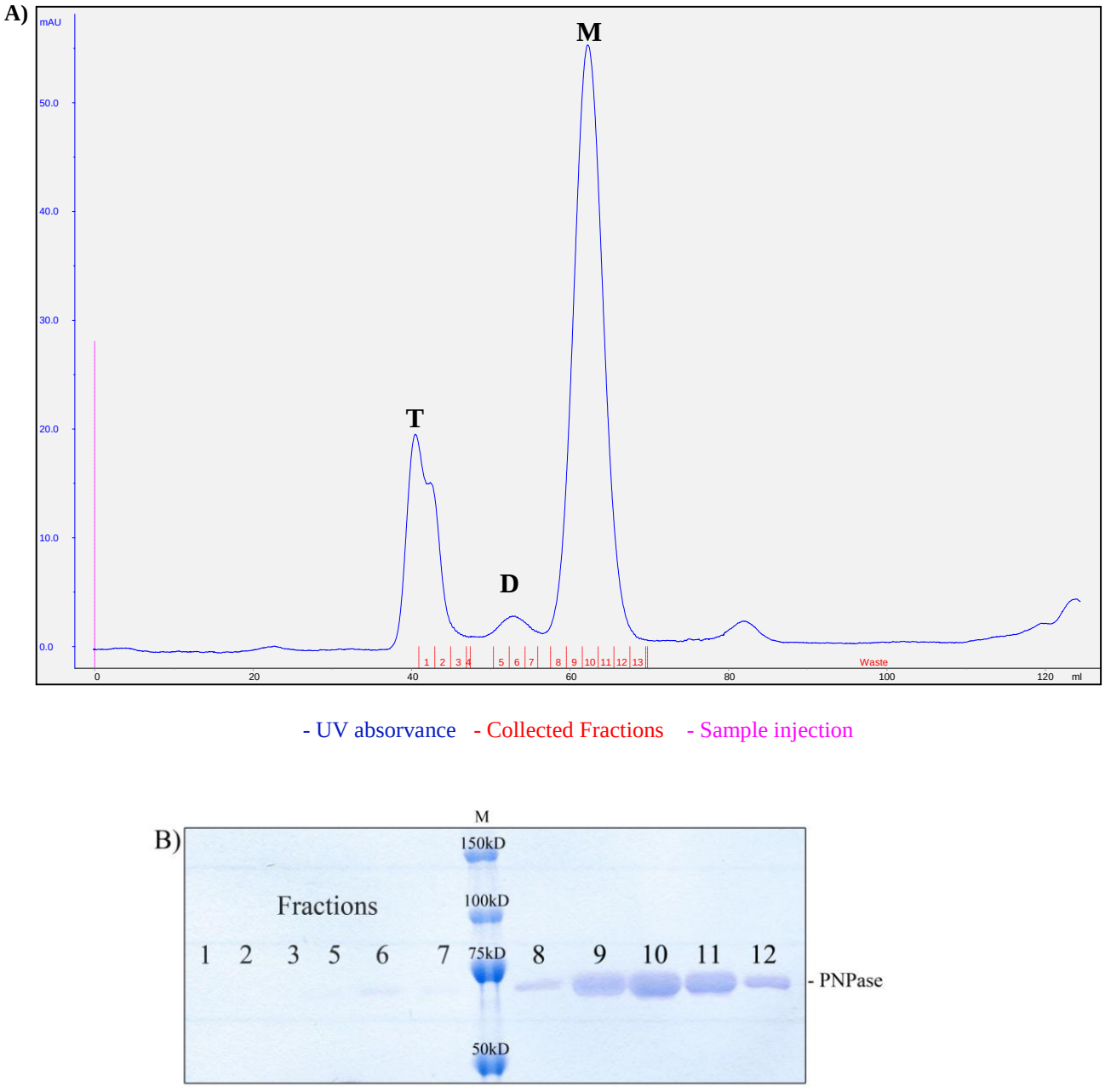


Figure 12: Gel filtration of *C. jejuni* PNPase_ΔS1KH. **A)** Chromatogram obtained in the protein purification. The first peak corresponds to PNPase_ΔS1KH in the trimeric form (T), the second corresponds to PNPase_ΔS1KH in the dimeric form (D) and the third corresponds to PNPase_ΔS1KH in the monomeric form (M). **B)** SDS-PAGE analysis of the fractions collected during gel filtration. Samples were denatured and separated in an SDS 8% polyacrylamide gel. Gel was stained with Comassie brilliant blue to visualize the protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented.

In order to confirm if PNPase wt and mutants are able to trimerize, the purified proteins were incubated with increasing concentrations of DSS (disuccinimidyl suberate).

In the presence of increasing concentrations of DSS, wild-type protein and the mutated versions of PNPase (PNPase_ΔS1 and PNPase_ΔS1KH) were able to form trimers at the higher concentration of DSS used (Figure 13). When the tested proteins were incubated with 1µg and 2µg of DSS, we can only observe a vestigial trimer formation (Figure 13).

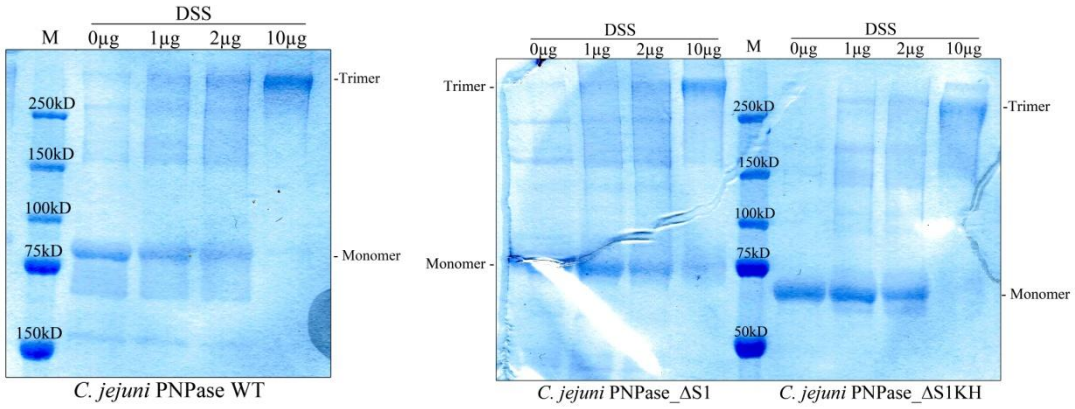


Figure 13: Cross-linking of *C. jejuni* PNPase wt and mutant proteins using DSS. 0,5 µg of purified proteins (*C. jejuni* wild-type PNPase, PNPase_ΔS1 and PNPase_ΔS1KH) were incubated with an increasing amount of DSS as indicated in the figure. After incubation, samples were denatured and separated in an 8% SDS-PAGE gel. Bands corresponding to PNPase trimer and monomer forms are indicated. Gel was stained with Comassie brilliant blue to visualize the protein bands. Lane M corresponds to a molecular-weight marker and respective sizes are represented above the bands.

3.2. *C. jejuni* PNPase *in vitro* assays

3.2.1. RNA binding ability

Electrophoretic mobility shift assays (EMSA) were performed with *C. jejuni* wild-type PNPase and its truncated derivatives to determine the effect of S1 and KH deletions on the affinity of the enzyme using a model substrate.

It was possible to see that all the proteins were able to form RNA-protein complexes. However, these complexes start to appear at different protein concentrations for each PNPase version. Full-length PNPase formed a visible complex with poly(A) substrate at a lower protein concentration than PNPase_ΔS1 and much lower than PNPase_ΔS1KH (Figure 14). The RNA-protein complexes formed by *C. jejuni* PNPase wt starts to be visible in the polyacrylamide gel at a concentration of 10nM of protein. The RNA-protein complex formed by PNPase_ΔS1 starts to be visible gel at a concentration of 25nM and the complex formed by PNPase_ΔS1KH is vestigial at a concentration of 200nM (Figure 14).

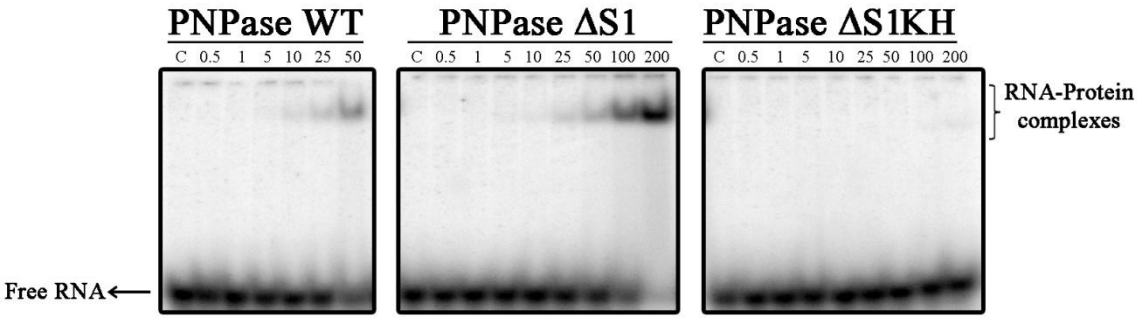


Figure 14: RNA binding activities of *C. jejuni* wild-type and mutated PNPases: 25nM of Poly(A) substrate was incubated for 10 minutes at 37 °C with increasing amounts of each protein at the concentrations indicated at the bottom of the panels (0,5nM to 200nM). Ctrl (Control) was performed with RNase free water instead of a purified enzyme.

3.2.2. Exoribonucleolytic activity assays

Purified *C. jejuni* wild-type PNPase, PNPase_ΔS1 and PNPase_ΔS1KH were assayed against a synthetic 35ss poly(A) substrate at 37°C.

All proteins exhibited significant PNPase activity as evidenced by the shortening of the marked substrate (Figure 15A). Also, they released an end-product of 4 nucleotides of length, except for ΔS1KH, which released a 2 nucleotides fragment (Figure 15A). Moreover and taking into consideration the concentrations used in this assay, it seemed that PNPase_ΔS1 was less active when compared to the wild type protein (figure 15A). PNPase_ΔS1KH was substantially less active when compared

with the other two proteins, since that, even when using 200nM, it was not able to completely cleave the poly(A) substrate (figure 15A).

Considering the results described above, the exoribonucleolytic activity of the three enzymes was determined by measuring and quantifying the disappearance of the substrate in several distinct experiments. For that, the protein concentration was adjusted so that less than 25% of substrate was degraded. Under those conditions, the activity was calculated as the amount of substrate (nM) degraded per nM of protein per minute. The results have shown that PNPase_ΔS1 is about 7-fold less active than the wild type, while the full C-terminal truncation, PNPase_ΔS1KH, exhibited approximately 0.1% of the activity of the wild-type enzyme (figure 15B).

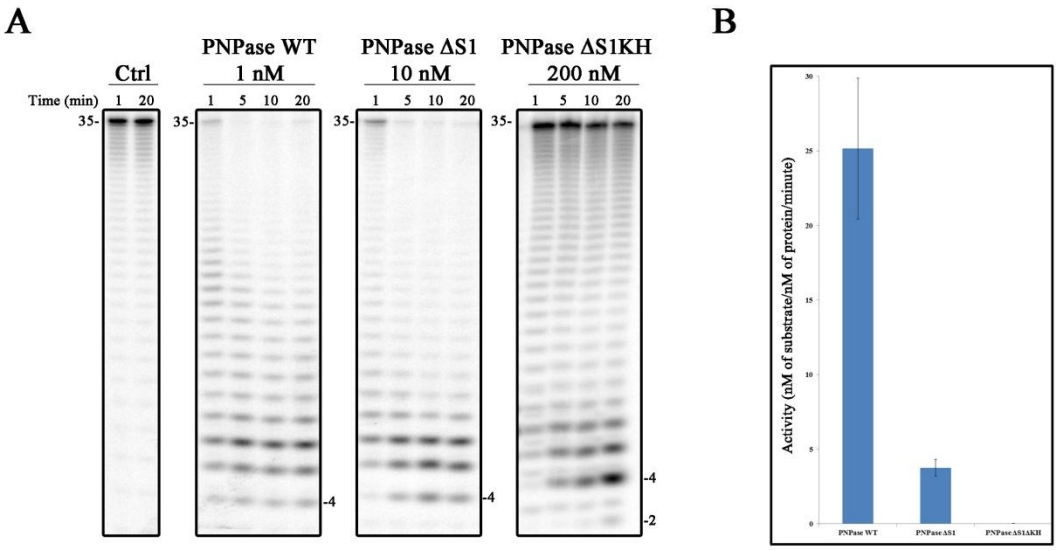


Figure 15: *C. jejuni* wild-type and mutated PNPase exoribonucleolytic activity: **A)** 1nM PNPase wt, 10nM PNPase_ΔS1 and 200nM PNPase_ΔS1KH were incubated with 15nM poly(A) substrate at 37°C for 20 minutes. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme. **B)** Determination of the activity of *C. jejuni* PNPase WT, PNPase_ΔS1 and PNPase_ΔS1KH, respectively. *Error bars* indicate the standard deviation.

Purified *C. jejuni* wild-type PNPase, PNPase_ΔS1 and PNPase_ΔS1KH were also assayed at four distinct temperatures: 4, 30, 37, and 42 °C. Those temperatures were chosen because 4°C is the temperature usually used for food storage; 30°C

because *C. jejuni* is not able to growth below this temperature (204); 37°C because it is the human internal temperature; and 42°C because it is the avian gut temperature, which is *C. jejuni* reservoir (195).

Either wild-type or truncated PNPase derivatives, PNPase_ΔS1 and PNPase_ΔS1KH, exhibited significant PNPase activity as evidenced by the shortening of the marked substrate at all assayed temperatures (figures 16A, 17A and 18A). The exoribonucleolytic activity of the three enzymes was also determined at the different temperatures, as described above (figure 16B, 17B and 18B).

Wild-type PNPase was shown to be more active at 37°C and 42°C (figure 16B). The exoribonucleolytic activity at those temperatures was very similar and is about ten and twenty times higher than the exoribonucleolytic activity at 30°C and at 4°C respectively (Figure 16B). Despite the similarities in the activity, at 42°C the wild-type PNPase seemed to be less efficient than at 37°C in degrading the RNA fragments of small size (Figure 16A). The same behavior was observed at 4°C and 30°C, and at these temperatures, the final product of 4 nucleotides was not obtained.

PNPase_ΔS1 exoribonucleolytic activity was shown to be higher at 37°C (Figure 17B). At 4°C and 30°C, the PNPase_ΔS1 showed significant less activity than at 37°C and 42°C. It was about 4-fold less active at 30°C, while at 4°C exhibited approximately 7% of the exoribonucleolytic activity (when compared with the maximum activity at 37°C) (Figure 17B). Contrary to what was observed for the wild type protein, at 42°C, PNPase_ΔS1 seemed to be more efficient in degrading the short RNA fragments (Figure 17A). In contrast, at 4º C and 30ºC, this protein was not able to completely degrade the poly(A) substrate until a final product of 4 nucleotides (Figure 17A).

Contrary to what was verified for wild-type PNPase and PNPase_ΔS1 (Figures 16 and 17), the activity of PNPase_ΔS1KH at different temperatures did not show remarkable differences (Figure 18B). The major difference was observed at 4°C, where the activity was approximately half when compared with the activity at the other temperatures.

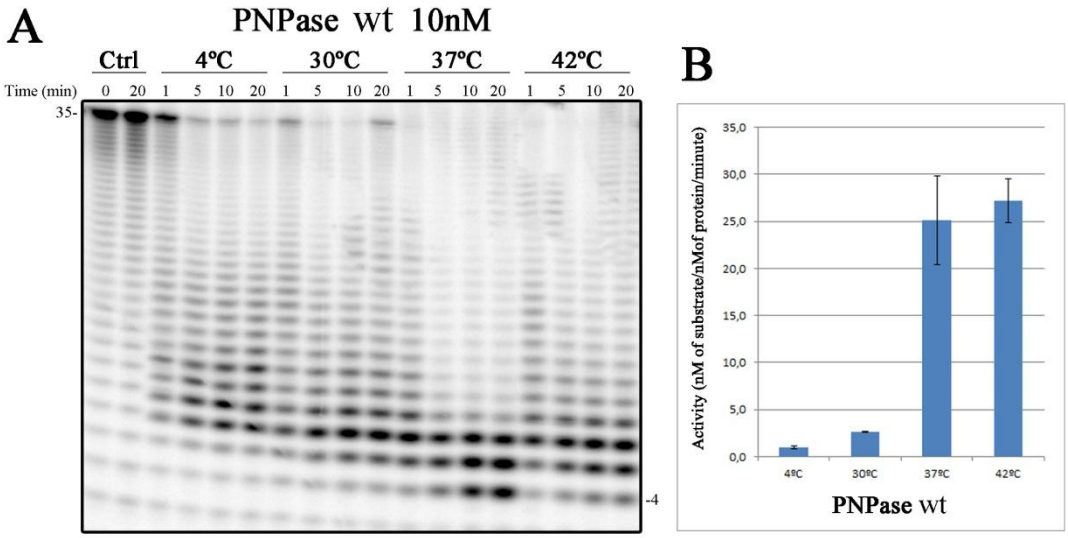


Figure 16: *C. jejuni* PNPase wt exoribonucleolytic activity at different temperatures**. A:** 10nM PNPase wt was incubated with 15nM poly(A) substrate at different temperatures (4; 30; 37 and 42°C) for 20 minutes. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme. **B:** Determination and comparison of the activity of *C. jejuni* PNPase wt at 4, 30, 37 and 42°C, respectively. *Error bars* indicate the standard deviation.

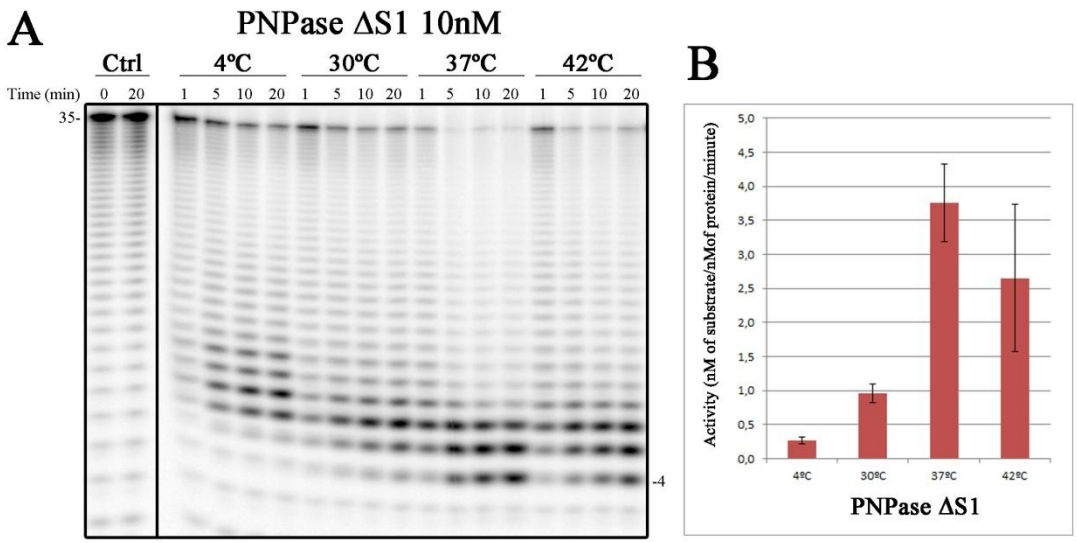


Figure 17: *C. jejuni* PNPase_ΔS1 exoribonucleolytic activity at different temperatures. **A:** 10nM PNPase_ΔS1 was incubated with 15nM poly(A) substrate at different temperatures (4; 30; 37 and 42°C) for 20 minutes. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme. **B:** Determination and comparison of the activity of *C. jejuni* PNPase_ΔS1 at 4, 30, 37 and 42°C, respectively. *Error bars* indicate the standard deviation.

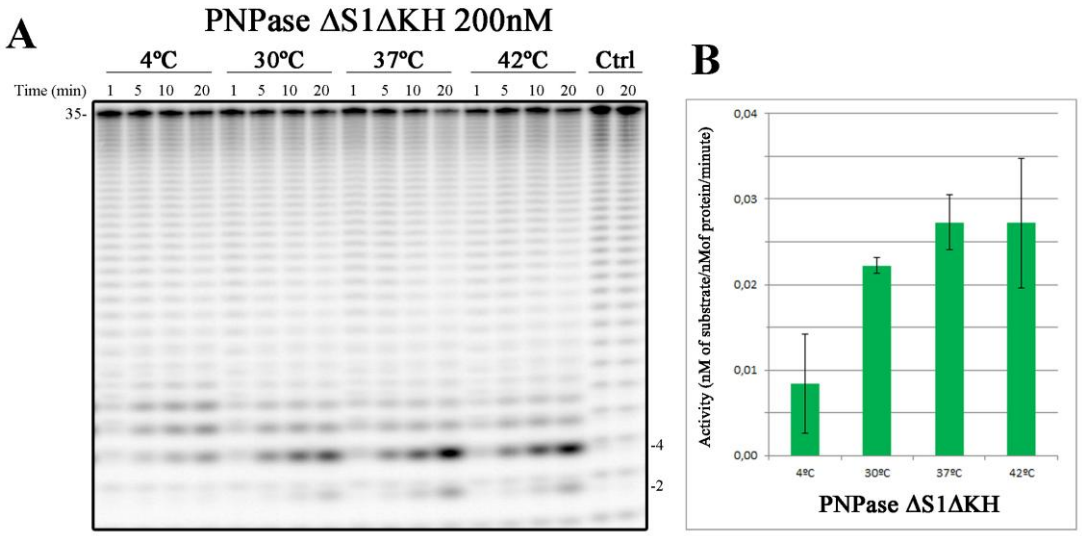


Figure 18: *C. jejuni* PNPase_ΔS1KH exoribonucleolytic activity at different temperatures. **A:** 10nM PNPase_ΔS1KH was incubated with 15nM poly(A) substrate at different temperatures (4; 30; 37 and 42°C) for 20 minutes. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme. **B:** Determination and comparison of the activity of *C. jejuni* PNPase_ΔS1KH at 4, 30, 37 and 42°C, respectively. *Error bars* indicate the standard deviation.

3.2.3. *C. jejuni* PNPase exoribonucleolytic activity using ssRNA or ssDNA substrate in the presence of two different divalent metal ions.

Purified *C. jejuni* wild-type PNPase was assayed against a ssRNA (poly(A)) and a ssDNA substrate. PNPase was incubated at two different concentrations at 37°C. For each concentration and for each substrate, the PNPase exoribonucleolytic activity was tested in the presence of two different divalent metal ions. Magnesium is the described co-factor for PNPase and it was the one used previously. However, catalysis can also occur in the presence of other divalent ions, such as manganese. It was already described that manganese is an example of a cofactor that could replace the role of magnesium in certain proteins (221). As such, we have also tested the exoribonucleolytic activity in the presence of manganese.

The results have showed that PNPase was efficient in degrading the ssRNA substrate in the presence of both divalent metal ions. In contrast, it did not show any activity on ssDNA substrate in the conditions tested (Figure 19).

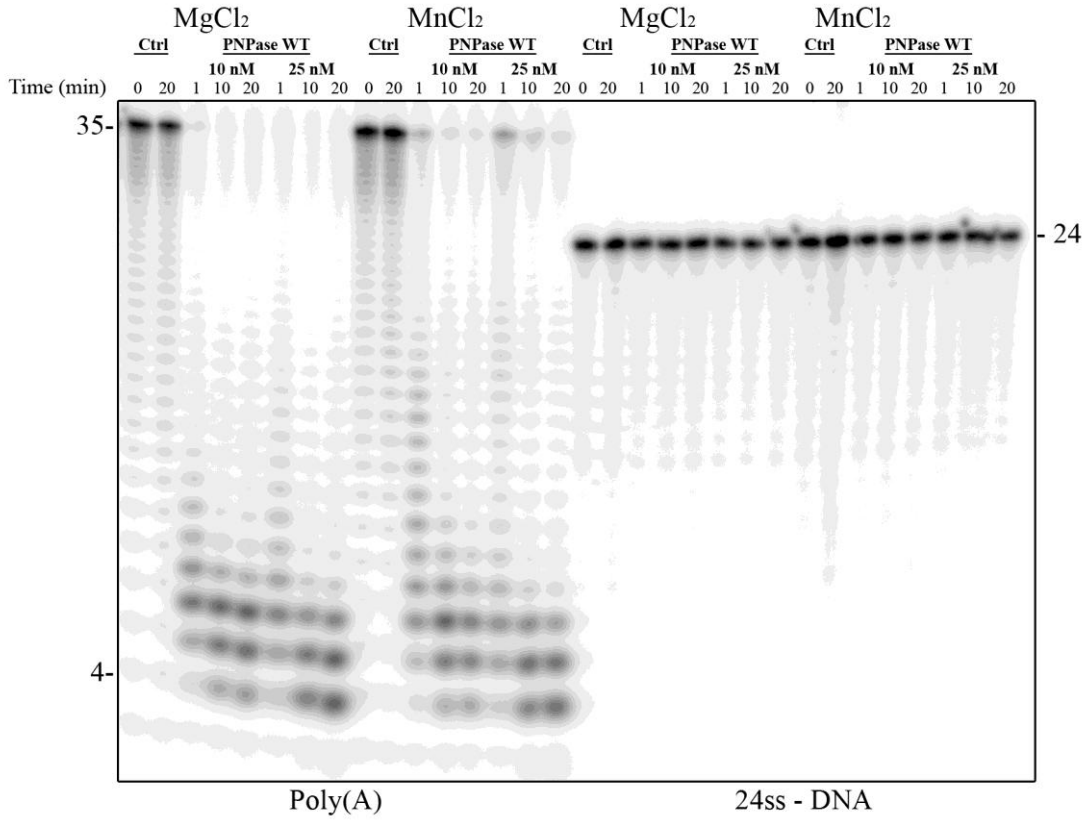


Figure 19: Divalent metal ion dependence of *C. jejuni* PNPase using ssRNA or ssDNA molecules as substrate. 10nM and 25nM of C. jejuni PNPase were incubated with 15nM of the two different substrates (poly(A) and 24ss DNA) at 37°C for 20 minutes in a reaction buffer with different divalent metal ions ($MgCl_2$ and $MnCl_2$), which are indicated in the figure. Samples were taken during the reaction at the time points indicated (1, 10 and 20 min). Ctrl (Control) was performed with RNase free water instead of purified enzyme.

3.2.4. *C. jejuni* PNPase exoribonucleolytic in the presence of different compounds

Since PNPase was shown to be quite relevant for virulence in *C. jejuni* it would be important to find compounds which modulate PNPase activity. Therefore, it was tested how *C. jejuni* PNPase exoribonucleolytic activity is affected in the presence of compounds that are implicated in the regulation of the cellular metabolic status. For that, we have performed activity assays in the presence of four compounds: ppGpp, ATP, citrate and cyclic di-GMP. All these compounds were good candidates since there

were few reports which linked them to PNPase (147,169,177,183). All the assays were performed in the same conditions (1 nM of purified enzyme and 15 nM of substrate).

ppGpp was tested in crescent concentrations of 0,1mM to 2,5mM. The results showed that it affected *C. jejuni* PNPase by decreasing the exoribonucleolytic activity. When used at a concentration of 0.1 mM, we could see that PNPase was still active; however the enzyme was unable to degrade the RNA smaller than 15 nucleotides. With the increase of ppGpp concentrations, the enzyme released larger RNA fragments, until the maximum concentration used (2,5 mM), in which the activity was completely abolished (Figure 20).

ATP was tested in crescent concentrations of 0,5mM to 50mM and it has also affected the exoribonucleolytic activity of *C. jejuni* PNPase, by reducing it. When used at a concentration of 0.5 mM, we could see that PNPase was still active; however the enzyme was unable to degrade RNA fragments smaller than 7 nucleotides. Similarly to what was observed in the presence of ppGpp, with higher ATP concentrations, the enzyme released larger RNA fragments. When present at a final concentration of 25 mM, ATP completely abolished PNPase activity (Figure 21).

Citrate was tested in a crescent concentration of 10mM to 100mM and it also reduced the *C. jejuni* PNPase exoribonucleolytic activity. When used at a concentration of 10 mM, we could see that PNPase was still active; however the enzyme was unable to degrade the RNA smaller than 10 nucleotides. With the increase of citrate concentrations, the enzyme released larger RNA fragments, until the maximum concentration used (100 mM), in which the enzyme was unable to degrade the RNA smaller than 16 nucleotides (Figure 22).

Cyclic di-GMP was tested in a crescent concentration of 0,1mM to 1mM and it also affected the *C. jejuni* PNPase exoribonucleolytic activity. It was possible to see an inhibitory effect of cyclic di-GMP, even in the lower concentration tested. When present at a concentration of 0.5 mM, we could see that PNPase activity was abolished almost to completion (Figure 23).

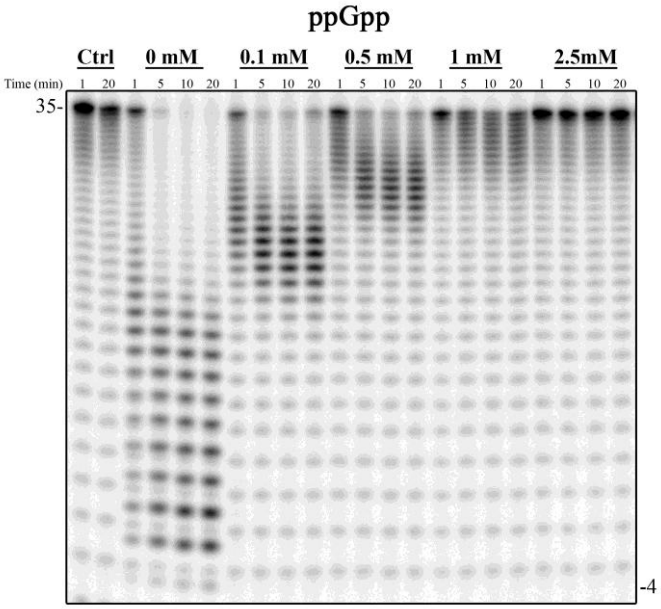


Figure 20: *C. jejuni* PNPase exoribonucleolytic activity in the presence of ppGpp. 1 nM PNPase wt was incubated with 15 nM poly(A) substrate at 37°C for 20 minutes in the presence of increasing concentrations of ppGpp (as indicated in the figure). Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme and 2,5mM of ppGpp.

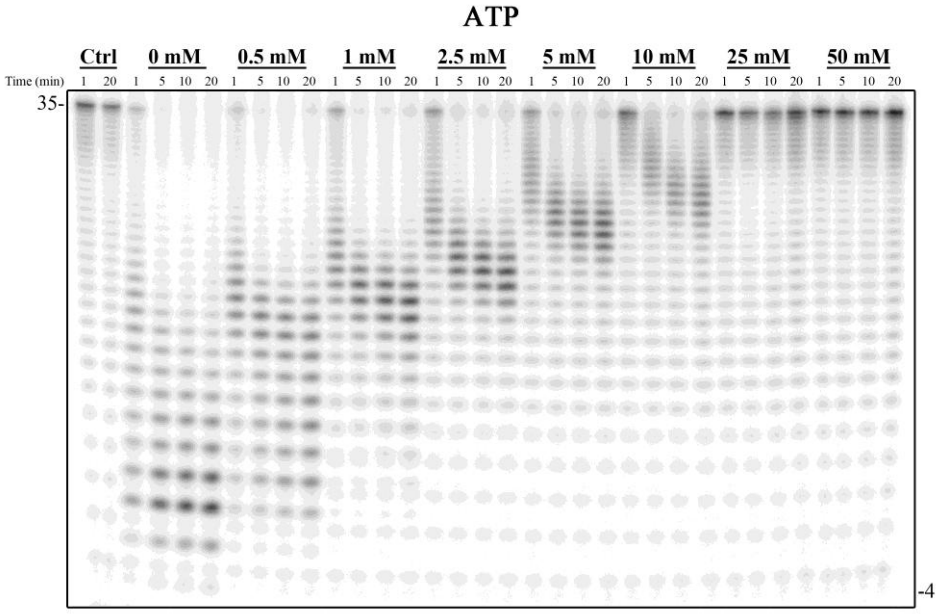


Figure 21: *C. jejuni* PNPase exoribonucleolytic activity in the presence of ATP**.** 1 nM PNPase wt was incubated with 15 nM poly(A) substrate at 37°C for 20 minutes in the presence of increasing concentrations of ATP (as indicated at the bottom of the panels (0 mM to 50 mM)). Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme and 50 mM of ATP.

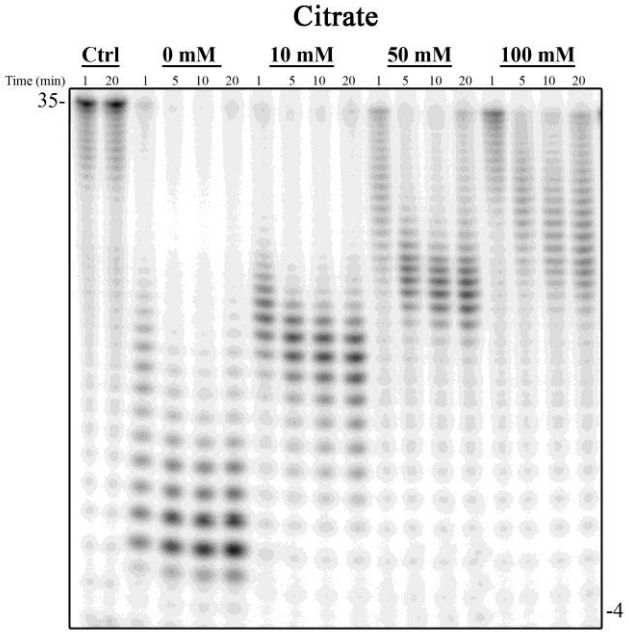


Figure 22: *C. jejuni* PNPase exoribonucleolytic activity in the presence of Citrate**.** 1 nM PNPase wt was incubated with 15 nM poly(A) substrate at 37°C for 20 minutes in the presence of increasing concentration of citrate (as indicated in the figure). Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme and 100 mM of citrate.

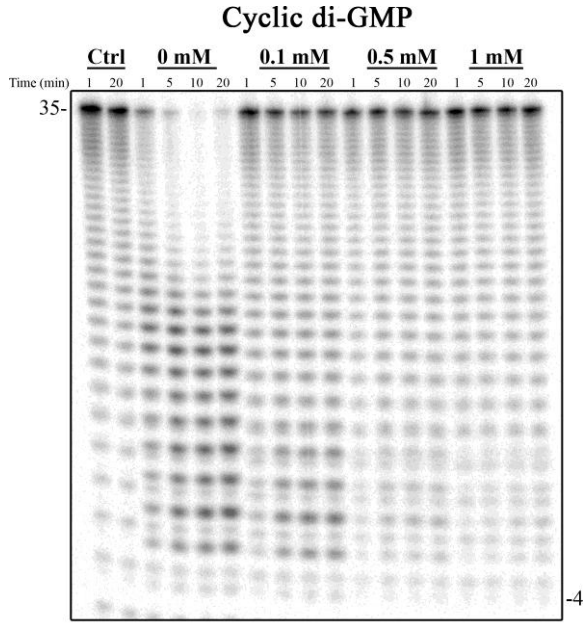


Figure 23: *C. jejuni* PNPase exoribonucleolytic activity in the presence of Cyclic di-GMP. 1 nM PNPase wt was incubated with 15 nM poly(A) substrate at 37°C for 20 minutes in the presence of increasing concentrations of cyclic di-GMP (as indicated in the figure). Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme and 1 mM cyclic di-GMP.

3.2.5. *C. jejuni* wild-type and mutated PNPase polymerization activity

In order to test the polymerization activity of *C. jejuni* PNPase *in vitro*, an activity assay was performed using the previously characterized 16ss RNA substrate and ADP in the absence of inorganic phosphate (Pi).

The assays were performed with the three versions of *C. jejuni* PNPase (wild-type, PNPase_ΔS1 and PNPase_ΔS1KH) in the presence of 1 mM ADP, and 15 nM substrate at 37ºC.

All the proteins exhibited polymerization activity as evidenced by the addition of single-stranded adenine-rich tails, enlarging the labeled substrate, and the disappearance of the 16ss substrate (Figure 24). Similarly to what was observed with the exoribonucleolytic activity, PNPase_ΔS1KH has shown less efficiency than the wild-type PNPase and PNPase_ΔS1 in the polymerization activity. In the wild-type PNPase and PNPase_ΔS1 it was possible to see well defined fragments in the top of the polyacrylamide gel, while with PNPase_ΔS1KH we could only observe a smear instead of defined bands, and the 16ss substrate did not disappear so rapidly (Figure 24).

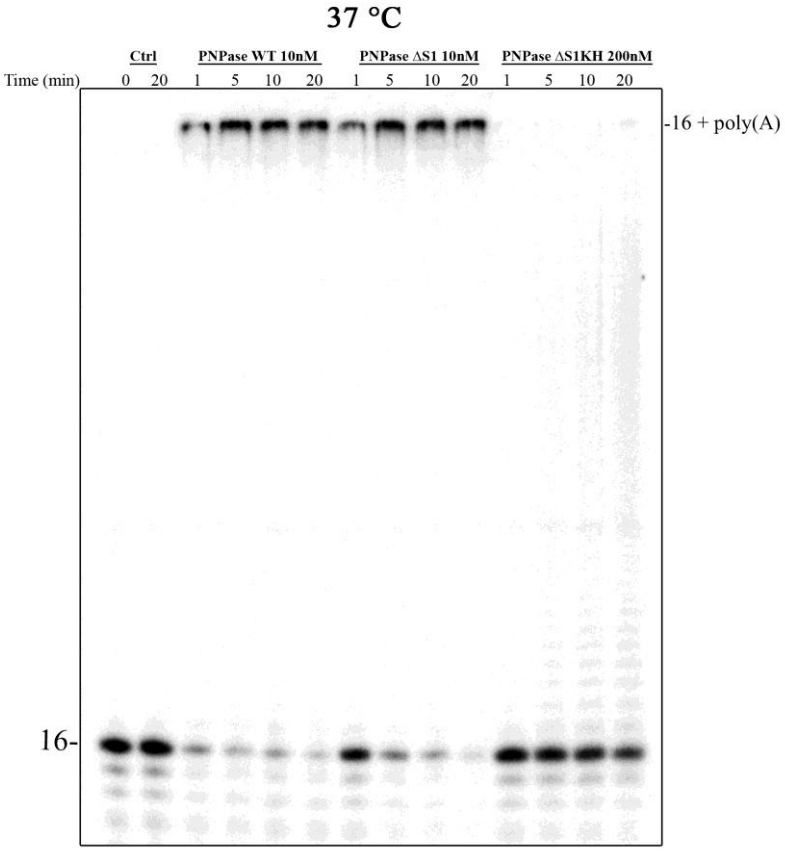


Figure 24: *C. jejuni* wild-type and mutated PNPases polymerization activity at 37°C**.** 10 nM PNPase wt, 10 nM PNPase_ΔS1 and 200 nM PNPase_ΔS1KH were incubated with 15 nM 16ss substrate at 37°C for 20 minutes in the presence of 1 mM ADP. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme.

The polymerization activity was also tested at other temperatures: 4ºC, 30ºC and 42ºC. Both wild-type PNPase and PNPase_ΔS1 exhibited the same significant activity at all the temperatures tested. However and in contrast with what was observed with the exoribonucleolytic activity, these proteins appeared to be more active at 30°C (Figure 26) than at 4°C (Figure 25) and 42°C (Figure 27). Comparing the abundance of the 16ss band of wild-type PNPase and PNPase_ΔS1 at 30°C (Figure 26) with the results obtained at 37°C, it was visible a major disappearance of the 16ss band at 37°C (Figure 24). This indicates that wild-type PNPase and PNPase_ΔS1 have more polymerization activity at 37°C than at the other tested temperatures. At 4°C (Figure 25), the

PNPase_ΔS1KH protein did not show any evidence of polymerization activity. At 30ºC and 42°C, it was only possible to see a vestigial activity for this protein (Figure 26 and Figure 27).

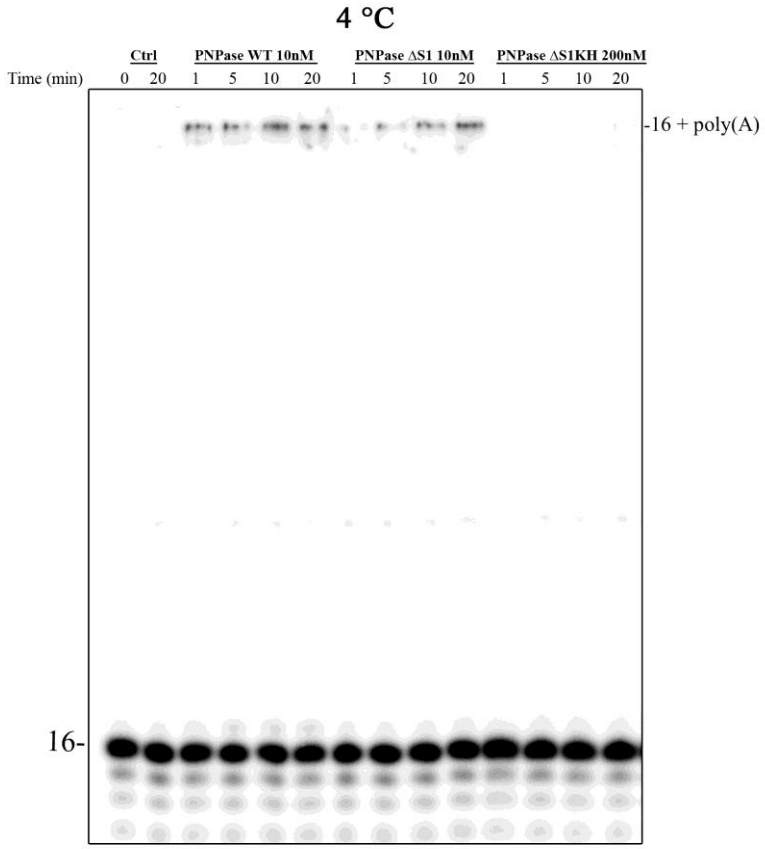


Figure 25: *C. jejuni* wild-type and mutated PNPase polymerization activity at 4°C. 10nM PNPase WT, 10nM PNPase_ΔS1 and 200nM PNPase_ΔS1KH were incubated with 15nM 16ss substrate at 4°C for 20 minutes in the presence 1mM ADP. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme.

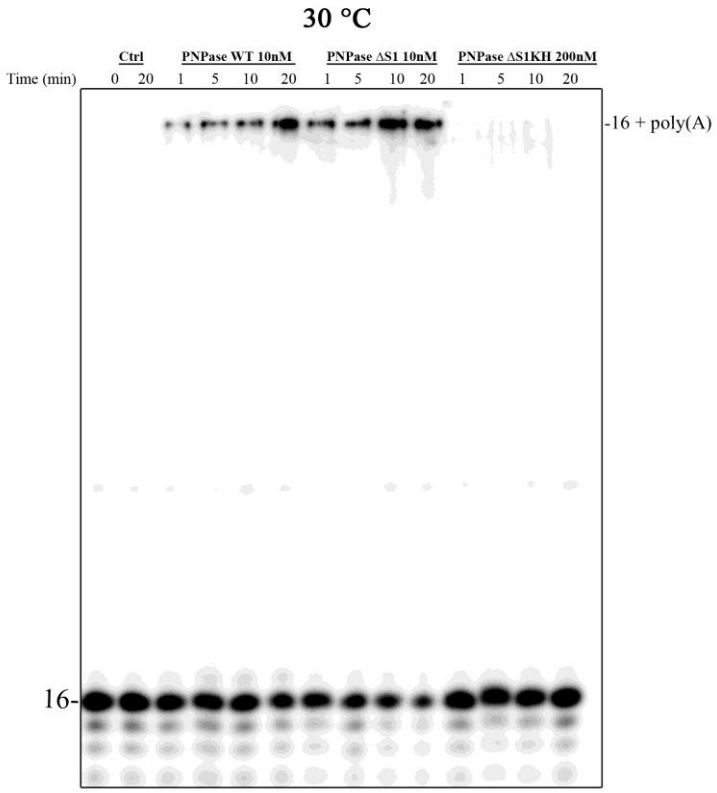


Figure 26: *C. jejuni* wild-type and mutated PNPase polymerization activity at 30°C. 10nM PNPase WT, 10nM PNPase_ΔS1 and 200nM PNPase_ΔS1KH were incubated with 15nM 16ss substrate at 30°C for 20 minutes in the presence 1mM ADP. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme.

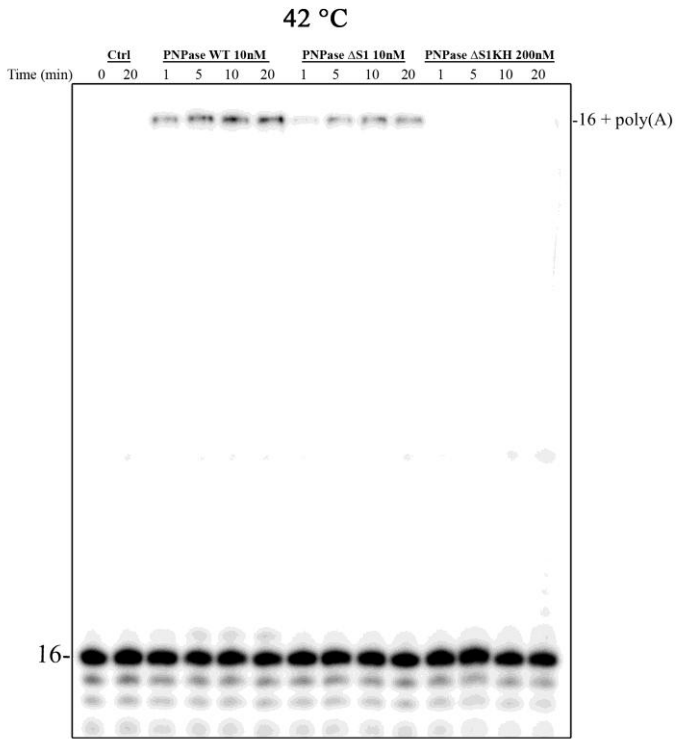


Figure 27: *C. jejuni* wild-type and mutated PNPase polymerization activity at 42°C**.** 10nM PNPase WT, 10nM PNPase_ΔS1 and 200nM PNPase_ΔS1KH were incubated with 15nM 16ss substrate at 42°C for 20 minutes in the presence 1mM ADP. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). Ctrl (Control) was performed with RNase free water instead of purified enzyme.

3.2.6. *C. jejuni* PNPase polymerization activity in the presence of different compounds

The effect of ppGpp, ATP, citrate and cyclic di-GMP in the polymerization activity of *C. jejuni* PNPase was also tested. The concentration of each compound used in these assays was the one that completely inhibit the PNPase exoribonucleolytic activity (Figures 20, 21, 22 and 23).

The results showed that ATP and citrate, in the concentrations used, were able to inhibit the polymerization activity of PNPase. However, in the presence of citrate, it was possible to see the addition of one or two nucleotides to the RNA substrate (Figure 28). In the presence of ppGpp it was possible to see that the polymerization activity was

also affected, although vestigial polymerization products were observed (Figure 28). Cyclic di-GMP did not seem to significantly affect the *C. jejuni* PNPase polymerization activity in the conditions tested (Figure 28).

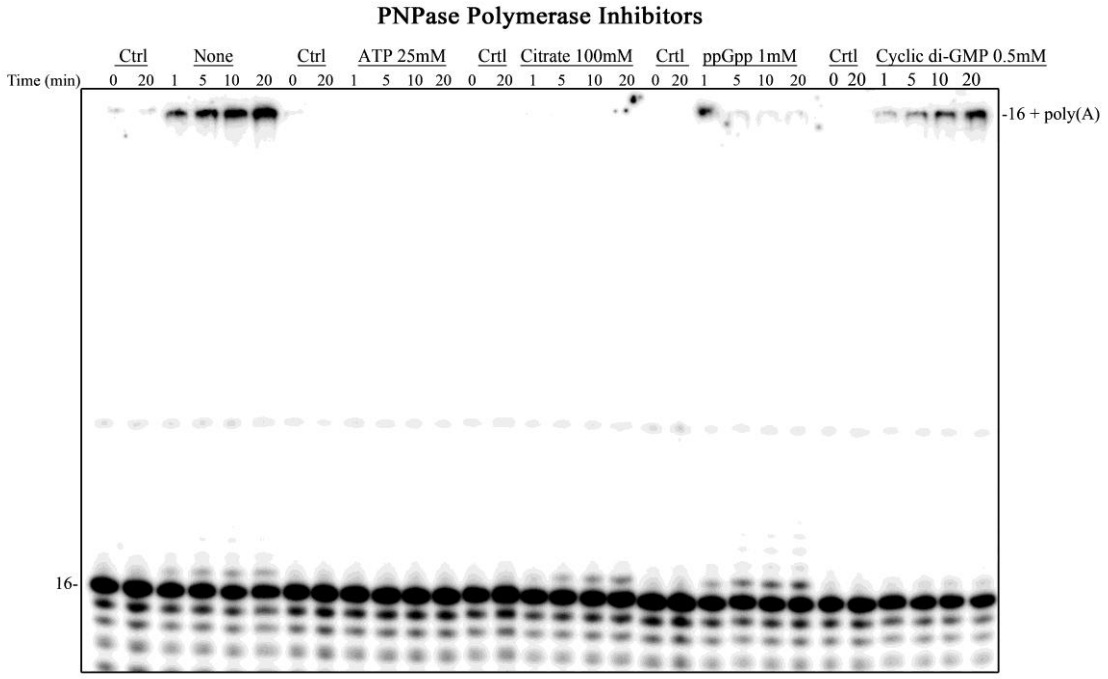


Figure 28: *C. jejuni* PNPase polymerization activity in the presence of certain compounds. 10 nM PNPase WT was incubated with 15 nM 16ss substrate at 37°C for 20 minutes in the presence of 1 mM ADP and each compound. Each compound and the respective concentration is indicated in the figure. Samples were taken during the reaction at the time points indicated (1, 5, 10 and 20 minutes). One Ctrl (Control) was performed for each different compound tested, with RNase free water instead of purified enzyme and with the respective compound.

4. Discussion and conclusion

C. jejuni is a foodborne bacterial pathogen that is now considered the leading cause of human bacterial gastroenteritis worldwide. Due to its clinical importance, it is important to have a more complete understanding about *C. jejuni* pathobiology, physiology, and regulation of the response mechanisms in order to develop new strategies to control *C. jejuni* (199,207,208).

This pathogen is contracted via the oral-fecal route. Its ability to persist and grow at refrigerated/cold temperatures is a major threat to public health considering that refrigeration is typically used to conserve food (168).

RNA metabolism remains poorly understood in this foodborne pathogen. However, Haddad et al. have studied *C. jejuni* PNPase, and have shown that this exoribonuclease is essential for low-temperature cell survival (166). They have also shown that PNPase deficiency induces swimming limitation, decrease of cell adhesion/invasion ability and delay in chick colonization (186). However, the mechanism by which PNPase can confer these features to *C. jejuni* is not yet understood.

In this work, *C. jejuni* wild-type PNPase and truncated versions of the protein were overexpressed and different biochemical analysis were performed to characterize them. It is very important to have a better understanding about the biochemistry of *C. jejuni* PNPase to know how it is influenced by physical and chemical factors in order to unravel the mechanism of action and discern its involvement in virulence.

4.1. RNA binding domains from PNPase are important for trimer formation

PNPase is a homotrimeric protein that exhibits a doughnut-shaped structure (150). In this work we have overexpressed and purified *C. jejuni* PNPase (wt and truncated versions). Since the expression was performed in *E. coli*, we have used the *E.coli* ENS134-3 strain (219) instead of a normal *E. coli* BL21(DE3). ENS134-3 strain lacks the endogenous PNPase, and avoids the purification of heterotrimers.

In order to verify if PNPase from *C. jejuni* also forms trimers, we have performed a gel filtration step after the first step of purification of the proteins. By

interpreting the chromatograms and SDS gels after purification of wild-type and truncated versions of PNPase it was possible to discern in which arrangement (trimers, dimers or monomers) and respective proportions we may have for each protein.

There are three peaks in each chromatogram obtained by gel filtration. The first one should correspond to a trimer, the second to a transient dimeric form, and the third to a monomer. By comparing the amplitude of each peak for each chromatogram we may have an idea of the proportion of each form. This evaluation is complemented by the SDS-PAGE analysis. As a control, we have also purified PNPase from *E. coli* (data not shown), which was already described as a trimeric protein (222).

The results of wild-type PNPase purification by gel filtration showed that this protein was, in majority, found in its trimeric form (Figure 10). This result was similar to the one obtained for *E. coli* protein. In the absence of the S1 binding domain, we could see that the purified protein was no longer predominantly in the trimeric form, as the wild-type protein, but mostly in the monomeric and dimeric state (Figure 11). When the protein lacks both binding domains (S1 and KH), the protein was found mainly in the monomeric form (Figure 12).

These results indicate that the lack of the S1 domain already imposes difficulties for the formation of the trimers, and this difficulty is increased when KH domain is also missing. Therefore, we can conclude that both S1 and KH domains are important for the trimeric structure of *C. jejuni* PNPase. Our results are in agreement with the ones presented in Amblar et al. (223). They have shown that the S1 domain from *E. coli* PNPase is able to induce the trimerization of the RNaseII-PNP hybrid protein, indicating that this domain can have a role in the formation of multimers.

In order to confirm these results, we have performed a cross-linking experiment in the presence of DSS (which is used to protein cross-linking to create bioconjugates via single-step reactions) (Figure 13). The cross-linking results have shown that wild type PNPase and both truncated forms of PNPase are able to trimerize. These results seem in disagreement with the ones obtained by gel filtration. However, while gel filtration was performed in real time, in the cross-linking reaction once the protein forms the dimer or trimer the reverse reaction cannot occur, what may happen in gel filtration. Ultimately, the cross linking experiments have shown that PNPase has the capacity of multimerization, even without the presence of the binding domains. The

same conclusion has already been reached when working with *E. coli* PNPase (149). Our results confirm that the S1and KH domains are not only involved in the RNA-binding (156), but they are also important for protein multimerization since they confer stability to the trimers.

4.2. S1 and KH domains from *C. jejuni* PNPase are important for RNA-binding

PNPase can be considering a multifunctional protein. Beside its role as a phosphorolytic exoribonuclease, it can also have polymerization and phosphate exchange activities (224).

PNPase is composed by a five-domain structure, organized in two N-terminal RNase PH domains connected by an α-helical linker, constituting the core domain (responsible for enzymatic activity), and two C-terminal domains, KH and S1, involved in RNA binding (Figure 1 and 2) (150). Stickney et al. (156) have shown that the deletion of S1 and KH domains from *E. coli* PNPase result in an accentuated loss of activity and RNA binding.

In order to characterize the activity of *C. jejuni* PNPase wild type and truncated versions, we have overexpressed and purified them, and performed activity and binding assays using synthetic RNAs previously described (208). Our results have shown that the lack of S1 result in a ~2,5-fold decrease of the efficiency in which the enzyme binds to the substrate (Figure 14). In the absence of both binding domains (S1 and KH), this decrease is more accentuated (more than 20-fold) (Figure 14). This data led us to conclude that, similarly to what was shown for *E. coli (156)*, *C. jejuni* PNPase domains S1 and KH are important for the RNA binding, since their deletion caused a reduction in the affinity of the enzyme for RNA. It was suggested that S1 domain is arranged with the KH domain, forming an RNA binding surface that facilitates substrate recognition. However, our results have also shown that, even in the absence of both RNA binding domains, the protein was able to form RNA-protein complexes, which suggests that the core domain also has the ability of binding to RNA, although with less efficiency.

4.3. *C. jejuni* PNPase is a 3'-5' exoribonuclease specific for RNA, which activity is affected by temperature changes

We have also tested the ribonucleolytic activity of *C. jejuni* PNPase. Taking into account that PNPase is a phosphorolytic enzyme, we have used phosphate in our activity buffer. The results presented in Figure 15 have shown that PNPase is able to degrade the RNA tested and confirmed that it is a processive exoribonuclease that releases a fragment with 4nt of length. Also, the preferred co-factor is Mg^{2+}, although PNPase was also shown to be active in the presence of Mn^{2+} (Figure 19). Moreover and contrary to what was show for *E. coli* (154) and *B. subtilis* PNPases (144), in *C. jejuni*, PNPase is specific for RNA and cannot degrade DNA in the conditions tested. This implies that, in *C. jejuni* PNPase is exclusively involved in RNA metabolism. At low inorganic phosphate concentrations (as described in (224)) PNPase has also proved to be efficient in adding polymeric tails to a RNA substrate. The results presented in Figure 24 confirmed that *C. jejuni* PNPase has polymerization activity.

The results obtained in this study have also shown that PNPase_ΔS1 and PNPase_ΔS1KH are less active than the wild type (Figure 15 and 24). Our data is in agreement with previous studies performed in *E. coli* (156). We can conclude that the binding domains (S1 and KH) are important for both exoribonucleolytic and polymerization activities. The effect that these domains have in the activity are related with the loss of affinity for RNA (Figure 14).

Like it was seen for *E. coli* (125), the binding domains from *C. jejuni* PNPase are not crucial for the activity of the enzyme, since PNPase_ΔS1KH still has residual activity. However, these domains considerably facilitate and enhance the activity of PNPase indirectly by promoting substrate binding.

Interestingly, in the PNPase_ΔS1ΔKH mutant the final product of degradation was a fragment of two and not four nucleotides (Figure 15 and 18). Shi et al. (149) have shown that the crystal structure of *E. coli* PNPase lacking the S1 and KH domains is more expanded, containing a slightly wider central channel when compared with the wild-type protein. This suggests that these domains help PNPase to assemble into a more compact trimer, regulating the channel size. This change on PNPase structure in the absence of the binding domains can be a possible explanation for the results that we

have obtained. By changing the structure and channel size, the truncated protein may have more affinity for smaller fragments.

C. jejuni, as a pathogen, can withstand different adverse conditions, like temperature changes. The optimal growth temperature is at a relatively high temperature (42°C), but their minimal growth temperature is in the range of 31 to 36°C (204), and growth ceases abruptly around 30°C (205). However even at a lower temperature (4°C) *C. jejuni* can still survive for several weeks (204).

As such and to have a better understanding how *C. jejun*i PNPase activity varies with temperature, we have tested the activity of wild-type PNPase and truncated versions at different temperatures: 4ºC, 30ºC, 37ºC and 42ºC (Figure 16, 17, 18, 24, 25, 26 and 27).

The results have shown that PNPase activity is largely affected by temperature changes. For the three proteins tested, the exoribonucleolytic activities were higher at 37ºC (human body temperature) (Figure 16) and 42ºC (chicken body temperature) (Figure 16). At 30ºC and 4ºC (Figure 16) there is a significant decrease of the exoribonucleolytic activity. This is a surprising result if we considered that for the other main exoribonuclease present in the cell, RNase R, the activity at these temperatures in only 50% reduced (208). Moreover, PNPase is important for the long-term survival of *C. jejuni* at refrigerated temperatures (166). This may imply that the activity of PNPase needs to be regulated according with the temperature in order to allow the survival of *C. jejuni*. With the decrease in temperature, cell metabolism is readjusted and transcription, RNA degradation and translation may be impaired (160).

4.4. *C. jejuni* PNPase activity is modulated by different metabolites

Some studies suggest that RNA degradation communicates with central metabolism. Nucleotide and other metabolites are known to modulate the activity of PNPase (169). This modulation can vary between enzymes of different microorganisms, whereas the same compound can inhibit or stimulate enzymatic activity (169,178,183). Here, we have shown that *C. jejuni* PNPase activity is affected in the presence of several compounds.

ATP was able to inhibit both phosphorolysis and polymerization activities (Figure 21 and 28). The same result was obtained for *E. coli* PNPase (147), while in *Nomomurea* sp. ATP does not exert any effect (178). It was demonstrated that *E. coli* PNPase has an ATP-binding site, distinct from the one where the RNA substrate binds (147). It is possible that this ATP binding site also exists in *C. jejuni* PNPase, although it seems not to be conserved in al bacterial species. Thus, ATP would bind to *C. jejuni* PNPase inhibiting both its phosphorolytic and polymerization activities. This suggests that PNPase would act mainly at low ATP concentrations, whereas other ribonucleases would play a more significant role at high energy charge. This regulation of PNPase by ATP connects RNA metabolism and the energy charge of the cell also in *C. jejuni*.

Our results show that also citrate, an intermediate in the Krebs cycle, has an impact on *C. jejuni* PNPase activity, by decreasing it (Figure 22 and 28). *In vitro*, PNPase activity depends on the presence of Mg^{2+} as a co-factor. In these experiments, we have used 5mM of Mg^{2+} in the activity buffer. In the presence of mM concentrations of citrate, this metabolite would be predominantly complexed with Mg^{2+}, sequestering it from the reaction and thus inhibiting PNPase activities (Figure 22 and 28). In *E. coli*, it was shown that PNPase can bind to both citrate and magnesium-citrate. When citrate is complexed with magnesium, it inhibits the enzymatic activity of *E. coli* PNPase. When was tested predominantly in a metal-free form, the degradation and polymerization of the substrate were enhanced (169). These results suggest that citrate may have two different binding sites: one where it acts as an inhibitor in the metal-bound form and the other where it acts as an activator in its metal-free state (169). We have performed some preliminary experiments also in the presence of magnesium-citrate but the results were not conclusive. However, we cannot discard the hypothesis of having the same type of regulation by citrate as shown for *E. coli*.

ppGpp is an alarmone (an intracellular signal molecule that is produced due to harsh environmental factors) that regulates gene expression in bacteria. It is known to bind to RNA polymerase, changing its promoter preference, and thus regulating transcription. It was demonstrated that in *Streptomyces* and *Nomomurea* ppGpp can also be linked with RNA degradation pathways by inhibiting PNPase (177,178). We have tested the activity of *C. jejuni* PNPase in the presence of ppGpp. The results have shown that *C. jejuni* PNPase activity is also inhibited in the presence of this molecule (Figure

20 and 28). An increase of ppGpp levels allow bacteria to survive under unfavorable conditions, by switching their metabolism from a "growth mode" to a "survival mode" (170). It makes sense that, when bacteria enter the survival mode, they need to save energy and avoid degradation of certain mRNAs. As such, by affecting the activity of PNPase, ppGpp will decrease mRNA degradation, allowing the cell to save energy.

c-di-GMP is a second messenger used in signal transduction. It is implicated in several important processes in the cell, such as biofilm formation, changes is motility and virulence. More recently, it was also shown to be involved in RNA metabolism by enhancing the activity of *E.coli* PNPase (183). On the other hand, our *in vitro* assays with purified *C. jejuni* PNPase have shown that c-di-GMP inhibits the exoribonucleolytic activity (Figure 23), and it doesn't seem to affect the polymerization activity (Figure 28). c-di-GMP is involved in changes in motility and in virulence; PNPase was also shown to be important for virulence and motility in *C. jejuni*; these processes may require changes in a subset of mRNAs and for that, c-di-GMP modulates PNPase in order to change gene expression. However, further studies are still necessary to clarify this correlation.

Taken together our results show that PNPase from *C. jejuni* is highly modulated by several metabolites, supporting a link between the cellular metabolic status and RNA metabolism. Metabolites will influence the function of ribonucleases, which will have a wide impact on many transcripts, regulating the cellular proteome and metabolome.

4.5. Final Conclusions

In this work we have characterized the activity of *C. jejuni* PNPase. We have shown that PNPase is a trimer, and that both S1 and KH domains have an important role in trimer formation.

C. jejuni PNPase was shown to have a processive 3' to 5' degradative activity, and the preferred co-factor was Mg^{2+}. Moreover, it was shown to be specific for RNA, contrary to what was shown for other PNPases in other organisms. The S1 and KH domains were shown to be important for RNA binding and, consequently, for the activity of the protein.

Finally, *C. jejuni* PNPase activity was shown to be modulated by several metabolites. This suggests a link between the cellular metabolic status and RNA metabolism.

4.6. Future Perspectives

Given the results obtained in this study, many other questions have been raised, suggesting the need to perform future experiments. Moreover, taking into consideration that we have only analyzed two activities of PNPase, we still have to characterize the ADP/Pi phosphoryl-exchange activity of *C. jejuni* PNPase.

It would be also interesting, by overlapping PCR, to construct some PNPase point mutants. By modifying certain conserved amino acids, it will be possible to have a better understanding of *C. jejuni* PNPase mode of action. Also, it would be important to address the role of these point mutations and the role of PNPase RNA-binding domains in *C. jejuni* biology. For that, we will insert *pnp*_ΔS1, *pnp*_ΔS1KH and the other mutations in pRY111 vector and transform *C. jejuni* derivative strain (Δ*pnp*) (166). Then, we will be able to perform some *in vivo* tests to see how those mutations influence the growth and certain *C. jejuni* virulence features (motility, adhesion and invasion).

Finally, we would like to test, *in vivo*, the metabolites that were able to modulate *C. jejuni* PNPase activity *in vitro*. By inhibiting or reducing the PNPase activity, we may reduce the virulence or the growth of *C. jejuni*. By doing that using natural compounds, it would be a very advantageous discovery for the food industry and consequently, for human health.

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6. Appendix

Appendix I – Materials

1. Culture Media, solutions and gels

Table 11: Culture media, solutions and gels used in the experimental procedures.

Luria Agar (LA) medium	**Luria Broth (LB) medium**
- 10 g Tryptone - 5 g Yeast Extract - 10 g NaCl - 10 g Agar - ddH_2O to 1000ml - Adjust pH to 7 and autoclave	- 10 g Tryptone - 5 g Yeast Extract - 10 g NaCl - ddH_2O to 1000ml - Adjust pH to 7 and autoclave
Terrific Broth (TB) medium	**TBE 10x**
- 12 g Tryptone - 24 g Yeast Extract - 9.4 g K_2HPO_4 - 2.2 g KH_2PO_4 - Adjust pH to 7.2 and autoclave	- 108 g Tris Base - 55 g Boric acid - 9.3g EDTA - ddH_2O to 1000ml
TFB1	**TFB2**
- 0.588 g potassium acetate (30 mM) - 0.294 g $CaCl_2$ (10 mM) - 2.0 g $MgCl_2$ (50 $mM_)$ - 2.42 g RbCl (100 mM) - 30ml glycerol (15% v/v) - ddH_2O to 200ml - Adjust pH to 5.8 with 1M acetic acid - Filtrate solution with 0.45µm filter	- 0.21 g MOPS (10 mM) - 1.1 g $CaCl_2$ (75 mM) - 0.121 g RbCl (10 mM) - 15ml glycerol (15% v/v) - ddH_2O to 100ml - Adjust pH to 6.5 with 1M NaOH - Filtrate solution with 0.45µm filter

Polyacrylamide gel (denaturing) 20%	**Polyacrylamide gel (non-denaturing) 10%**
- 35ml 40% Acrylamide (19:1 acrylamide/bisacrylamide) - 29.4 g Urea - 7ml TBE 10x - Dissolve the urea at 60°C - Add ddH_2O to 70 ml and degas <u>Polymerisation of acrylamide is iniated by addition of:</u> - 300 µl APS 10% - 50 µl TEMED	- 15 ml 40% Acrylamide (19:1 acrylamide/bisacrylamide) - 6 ml TBE 10x - 39 ml H_2O <u>Polymerisation of acrylamide is iniated by addition of:</u> - 600 µl APS 10% - 60 µl TEMED
SDS-PAGE - Stacking gel	**SDS-PAGE - Running gel**
- 375 µl 40% Acrylamide (37:1 acrylamide/bisacrylamide) - 375 µl Tris 1.5M pH=6.8 - 30 µl SDS 10% - 2.2 ml H_2O <u>Polymerisation of acrylamide is iniated by addition of:</u> - 15 µl APS 10% - 5 µl TEMED	- 1.2ml 40% Acrylamide (37:1 acrylamide/bisacrylamide) - 1.52ml Tris 1.5M pH=8.8 - 60µl SDS 10% - 3.195ml H_2O <u>Polymerisation of acrylamide is iniated by addition of:</u> - 18 µl APS 10% - 8 µl TEMED
SDS-PAGE running buffer 10x	**Staining solution (Comassie brilliant blue)**
- 12.10 g Tris base - 40 g NaCl - H_2O MQ to 500 ml - Adjust pH to 7.6 with HCl	- 0.25 g *Coomassie Brilliant Blue* R-250 - 10 ml Acetic acid - 45 ml Methanol - 45 ml H_2O
Destaining solution	**Binding buffer 5x**
- 7.5 ml Acetic acid - 20 ml Methanol - 72.5 ml H_2O	- 10 µl Tris-HCl pH=8 (1M) - 25 µl EDTA (0.5M) - 30 µl KCl (1M) - 5 µl DTT (100mM) - 30 µl H_2O

Dilution buffer	**Exoribonucleolytic activity buffer PNPase 5x**
- 985 µl H_2O - 5 µl Tris pH=8 (1M) - 2 µl DTT (100mM) - 6 µl KCl (1M) - 2 µl NaH_2PO_4 (0.5M)	- 17.25 µl H_2O - 7.5 µl Tris pH=8 (1M) - 5 µl DTT (100mM) - 0.25 µl $MgCl_2$ (1M) - 15 µl KCl (1M) - 5 µl NaH_2PO_4 (0.5M)
Polymerization activity buffer PNPase 5x	**Buffer A**
- 11.75 µl H_2O - 7.5 µl Tris pH=8 (1M) - 5 µl DTT (100mM) - 0.25 µl $MgCl_2$ (1M) - 15 µl KCl (1M) - 0.5 µl ADP (500mM)	- 20 mM Tris-HCl pH=8 - 500 mM NaCl - 20 mM Imidazole - 2 mM β-mercaptoethanol
Buffer B	**Buffer C**
- 20 mM Tris-HCl pH=8 - 500 mM NaCl - 500 mM Imidazole - 2 mM β-mercaptoethanol	- 30 mM Tris-HCl pH=8 - 60 mM KCl - 10 mM NaH_2PO_4 - 1 mM $MgCl_2$ - 2 mM DTT
0.7% Agarose gel	
- 0.7 g Agarose - 100 ml TBE 1x - 20 µl ethidium bromide	

2. *E. coli* strains

Table 12: Strains used in the experimental procedures.

Strains	Relevant Genotype	Reference, source
DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK^-, mK^+) *pho*A *sup*E44 λ *thi*-1 *gyr*A96 *rel*A1	Invitrogen™
ENS134-3	BL21(DE3) (*lacZ*::Tn*10* *malP*pΔ534::P_{T7}*lacZ Arg5*)(*pnp*::Tn*5*)	(219) Kindly obtained from Marc Dreyfus, École Normale Supérieure, Paris, France

Appendix II – Primers and Sequences

1. Oligos used in this study

Table 13: Primers used in *pnp*_ΔS1 and *pnp*_ΔS1KH amplification.

Primers	Sequence (5' – 3')
Histag_FW	GCAGCGGCCATATCGACGAC
DeltaS1_Rev	GCCTGATGGATCCTAGTCTTTAGAGCC
DeltaK1S1_Rev	CGCTAAAAGGATCCAGTTAAGGTAAAAC

Table 14: RNAs used in activity assays.

RNAs used	Sequence (5' – 3')
Poly (A)	AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
16ss	CCCGACACCAACCACU

Table 15: DNA used in activity assays.

DNA used	Sequence (5' – 3')
24ss	AATATGGCTCATAGGCGCAGAGGG